FINAL PERFORMANCE REPORT

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Establishment of a Viable Headstart and Release Program for Texas Horned Lizards (*Phrynosoma cornutum*) in Oklahoma

Oklahoma Department of Wildlife Conservation

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Executive Summary:

Headstart programs, in which juvenile animals are reared in captivity during the life stage that experiences the highest mortality rates and then subsequently released into a managed wild setting as subadults or adults, are being developed with increasing frequency as a conservation tool for rare and declining species. This project developed and evaluated the first long-duration (eight months or longer) headstart program for the Texas Horned Lizard (*Phrynosoma cornutum*), which is a species of conservation concern throughout most of its range. This effort was built onto a 2019 pilot study and between 2020 to 2024, the project focused on captive-rearing, reintroduction, and post-release monitoring of Texas Horned Lizards at Tinker Air Force Base (TAFB), where an apparently isolated urban population has been studied for nearly 25 years. Horned lizards were hatched from wild-collected eggs or captured as recently emerged hatchlings and were raised to adult (20-month-old) and juvenile (8-month-old) lizards at the Oklahoma City Zoo's *Lizard Lab* and were released back on to TAFB to augment the existing population. Post-release tracking revealed that juvenile lizards demonstrated movement and survival rates comparable to their wild counterparts, while adult headstarted lizards (those released a 20+ months of age) exhibited smaller home ranges and higher mortality rates due to predation than their wild-reared counterparts. Digestive tract microbiome analysis showed a

transition from captive to wild microbial communities post-release, providing insights into lizard adaptation and health in natural environments.

During the grant period, 31 hatchling Texas Horned Lizards were successfully reared to adulthood in two cohorts and 27 hatchlings were reared to juveniles that were eight to nine months old in two other cohorts. All of these lizards were raised at the Oklahoma City Zoo and released back into their source population at Tinker Air Force Base (TAFB). Released juveniles exhibited comparable movement and survival rates to wild counterparts, while adults displayed smaller home ranges and lower survival rates, with predation being the primary cause of mortality. Most of the headstarted lizards adapted quickly to their new environment following their release, foraged effectively, and gained weight. Lizards also demonstrated natural brumation behavior during the fall following their release. At least two individuals, released as adults in 2023, exhibited natural reproductive behaviors in 2024, suggesting the program's potential contribution to population stability. Pre-release captive lizards exhibited distinct microbial communities that transitioned toward wild-type microbiomes within a few months following their release and indicating adaptation to the natural environment. Many released adults clustered near the release site through the summer following their release and a mass predation event significantly impacted survival, highlighting the need for refined release strategies. Based on the results of this study, we offer the following recommendations to improve the survival rates of future headstart releases. Soft-release durations should not be longer than three weeks and should begin in May to avoid potential periods of excessively warm and dry weather conditions. Additional measures, such as predator deterrents or strategic release site selection, should be implemented to reduce predation risk. Continued post-release tracking is crucial to evaluate long-term success and inform future conservation efforts. Educational outreach and conservation partnerships should be expanded to support habitat protection and awareness of Texas Horned Lizard conservation needs. This project marks a significant step toward maintaining viable Texas Horned Lizard populations in fragmented habitats in Oklahoma. The results from this study emphasize the importance of refining headstart rearing and release methodologies, including predator mitigation strategies, optimal release timing, and improved habitat selection.

Background and Need:

The Texas Horned Lizard, *Phrynosoma cornutum*, is one of the most charismatic and enigmatic reptile species of the American Southwest. Well known for its distinctive horns, lateral spikes, and flattened body, *P. cornutum* can be found in desert and grassland habitats with sandy or loose, loamy soils that facilitate burrowing for nesting and hibernation (Price, 1990; Burrow et al., 2001). The species requires mosaic habitat of open and shaded areas in order to thermoregulate throughout the day and between seasons (Bogosian 2010, Bogosian et al., 2012; Wolf et al., 2015), with home ranges shown to vary greatly in size (<100 –8,400m^2) based on a number of factors, including age, sex, and season (Fair and Henke, 1998). With an historic range covering parts of Arizona, Colorado, Kansas, Missouri, New Mexico, Oklahoma, Texas, and northern Mexico, the species has experienced a long-term range contraction and population decline spanning more than six decades (Price, 1990; Carpenter et al., 1993). These declines have been linked to a number of factors, including: (1) historic overexploitation for the pet trade (particularly in the first half of the 20th century), (2) the spread of invasive ants such as the imported red fire ant (*Solenopsis invicta*), (3) the loss of grassland habitats and conversion of grasslands to pastures of non-native grasses, (4) human expansion into the plains, which has resulted in habitat fragmentation, population isolation, and increased road mortality, and (5) a reduction in the abundance of their primary food source, harvester ants (*Pogonomyrmex* spp.) (Price, 1990; Carpenter et al., 1993; Donaldson et al., 1994, Howery 1996).

Due to increasing concerns regarding population level declines for *P. cornutum*, many states have introduced regulations to limit collecting. In Oklahoma, the Oklahoma Department of Wildlife Conservation (ODWC) has established a year-round closed season for the Texas Horned Lizard, which prohibits the capture, possession, and killing of the species. Oklahoma also has designated the Texas Horned Lizard as a Tier I "Species of Greatest Conservation Need" (SGCN) (Appendix E: Oklahoma Comprehensive Wildlife Conservation Strategy) and a Species of Special Concern Category II (ODWC 2025). The Texas Horned Lizard is state-listed as a threatened species in the neighboring state of Texas (TPW, 2018). To date, historical survey efforts have provided a baseline understanding of the species' distribution in Oklahoma. Despite these protections, populations in the eastern parts of the state continue to decline or have become locally extirpated (Moody, 2020). Although the species has managed to persist in small, often isolated pockets, as human encroachment continues it is critical that increased efforts be undertaken to monitor population health in Oklahoma and proactively address continued declines.

One example of a proactive measure taken in times of great concern over declining population trends is conservation translocation, involving deliberate movement and release of organisms to meet defined conservation objectives (IUCN/SSC, 2013). Among translocation initiatives, headstarting initiatives have been employed in a growing number of wildlife management strategies to reinforce population numbers for threatened wild populations (Redford et al., 2012; McGowan et al., 2017). The goal of headstart programs is to increase hatching and juvenile survival rates by rearing individuals in controlled, safe environments in captivity during early, vulnerable life stages before introducing individuals back into the wild as older, healthy subadults (Alberts, 2007, Haskell et. al. 1996, Mullin et. al. 2023). Such efforts are expected to increase wild population numbers by decreasing mortality events caused by a number of factors (i.e. predation and anthropogenic pressures) during developmental (nest incubation), hatchling, and sometimes juvenile life stages (Alberts and Philips, 2004; Alberts, 2007). Although a number of headstart programs employed to date suffer from inadequate monitoring and robust evaluation of long-term viability and success (Germano and Bishop, 2009; Brown et al., In review), they continue to serve an important role in population management and wildlife recovery plans, including efforts to combat declining population trends observed for *P. cornutum*.

Currently, several zoos in Texas have established headstart and reintroduction programs to supplement wild populations of Texas Horned Lizards, with the Fort Worth Zoo leading the way with a breeding program going back almost 20 years (Fort Worth Zoo, 2019). More recently, both the Dallas Zoo (Scudder, 2018) and the San Antonio Zoo (Underwood, 2019) have begun increasing reintroduction efforts as well. Unfortunately, the published data available on the long-term viability and success of these programs is limited, and there remains a paucity of information on a number of critical factors relating to their long-term viability and conservation impact. First, the existing *P. cornutum* headstart programs often release animals within the first eight weeks of life (the most vulnerable life stage for predation; Endriss et al., 2007) and do not have any tracking data post-release on reintroduction success rates, population establishment of reintroduced lizards, and headstart animal fecundity in the wild (Scudder, 2018; Fort Worth Zoo, 2019; Underwood, 2019). Second, all three Texas headstart programs are working with local populations of *P. cornutum* from Texas only. This is significant as there are clinal behavioral and morphological differences between populations of horned lizards in Oklahoma and Texas, with Oklahoma *P. cornutum* recognized as being smaller, having a shorter active season, and having lower reproductive rates possibly related to their smaller body size (Endriss et al., 2007). Therefore, it is unclear how applicable headstart protocols used on Texas populations of *P. cornutum* are to conservation efforts in Oklahoma. It is important that we develop and implement a program for improved protection and monitoring of locally adapted populations in Oklahoma while proactively exploring means to supplement declining populations across the state.

In Oklahoma, the most well-studied population of *P. cornutum* occurs at Tinker Air Force Base (TAFB), in Oklahoma Co., with published works and ongoing research covering population demographics, survival rates, habitat requirements, and general behavior dating back nearly 20 years (Endriss et al., 2007; Moody et al., 2007; Bogosian et al., 2012; Wolf et al., 2013, 2014, 2015; Mook et al., 2017). Since the start of horned lizard studies at TAFB in 2003, overall population size has declined, individual survival rates at all life stages have decreased, and small-scale habitat restoration efforts have shown little effect (Endriss et al., 2007; Wolf et al., 2013). To bolster this diminished urban population, the development of a new headstart program for Texas horned lizards was initiated in 2019 with the help of the Oklahoma City Zoo and with the explicit goal of developing a viable captive-rearing program to increase hatchling survivorship by holding them in captivity during early development (Endriss et al., 2007) and subsequently releasing subadult and adult lizards back into the source populations.

To increase our understanding of Texas horned lizard ecology and population-level genetic diversity, and to establish optimized best practice protocols for reintroduction efforts, we have included a novel microbiome component into the Oklahoma City Zoo headstart program. Microbiomes, communities of microscopic organisms living on the skin and in the gut of all vertebrates, are critical to host health. These communities aid in host nutrient acquisition, hormonal balance, and even immune response (Fraune & Bosch 2010). Interestingly, animals housed in captivity have been shown to possess distinct microbial communities and decreased microbial diversity when compared to wild counterparts (Zhu et al., 2011; Eigeland et al., 2012; Alfano et al., 2015; Cheng et al., 2015; Clayton et al., 2016). It has been speculated that headstart animals raised in captivity and released into the wild at later life stages may be hindered as a result of decreased microbial diversity; however, no published study has monitored microbial communities in headstart animals both pre- and post-release (Redford et al., 2012; Jimenez & Sommer, 2017; West et al., 2019). Through continuous sampling of microbial communities in headstart *Phrynosoma cornutum* before and after reintroduction, we will be able to determine if certain microbial strains or composition of the whole microbial community are correlated with increased or decreased host survivorship in the wild. Further, such sampling would address the extent to which headstart animals can acquire microbial diversity post-release, and how quickly.

Data regarding population-level genetic differences within Texas horned lizards are non-existent in Oklahoma, and only a few small-scale genetic studies have been conducted on populations in Texas (Guerra, 1998; Sattler and Ries, 1998; Williams et al., 2022; Wall, 2024). Establishing a baseline condition for the population genetic diversity and structure of wild *P. cornutum* at TAFB is desirable and headstart animals reared in captivity could inform appropriate conservation strategies for TAFB and state populations. This is particularly important for small and declining populations that are at risk of undergoing genetic bottlenecks

(Sattler and Ries, 1998) and experiencing inbreeding depression (Charlesworth and Willis, 2009). Together, these genetic and microbial datasets would be important for adjusting captive husbandry conditions to promote beneficial microbial assemblages through the use of probiotics or diet supplementation, and to maximize population genetic diversity, that could ultimately improve horned lizard survivorship in the wild (Redford et al., 2012; West et al. 2019).

This grant builds on to the work that was launched as a pilot study in 2019 to develop the Oklahoma City Zoo's *Lizard Lab*, which is the state's first headstart and reintroduction program for Texas Horned Lizards. This facility is capable of hatching eggs collected from wild populations, raising and maintaining lizards until wild release, and monitoring lizard health pre- and post-release to assess survivorship and improve programmatic success. The current grant was designed to continue the operation of the Oklahoma City Zoo's *Lizard Lab* and headstart program for Texas Horned Lizards and to explore the following five goals that were identified by a collaborative team comprised by the Oklahoma City Zoo, Sam Noble Museum, Tinker AFB, and ODWC:

1. Establish a best practice husbandry protocol and animal reintroduction plan for the development of *P. cornutum* headstart programs.
2. Develop and optimize protocols for supplementing isolated and declining populations to ensure the long-term viability of *P. cornutum* in mosaic landscapes across the state.
3. Establish baseline information on survivorship and growth rates of headstarted horned lizards into native environments.
4. Determine the speed and extent to which headstart animal gut microbial communities change post-release and establish baseline information on bacterial communities correlated with increased or decreased survivorship in the wild.
5. Determine and compare the patterns of genetic diversity between the TAFB and captive populations of *P. cornutum*, and its implications for the long-term viability of the headstart initiative.

Objectives:

***Objective 1*—**Establish a best practice husbandry protocol and animal reintroduction plan for the development of a *P. cornutum* headstart program.

***Objective 2*—**Establish baseline information on survivorship and growth rates of headstarted horned lizards into native environments.

***Objective 3*—**Determine the speed and extent to which headstart animal gut microbial communities change post-release and establish baseline information on bacterial communities correlated with increased or decreased survivorship in the wild.

***Objective 4*—**Analyze population genetic diversity of wild and captive horned lizards to assess the long-term genetic viability of the TAFB population.

Methods:

ANIMAL COLLECTION IN THE WILD AND REARING IN CAPTIVITY (OBJECTIVE 1)

Nest Identification:

Adult Texas Horned Lizards on TAFB have been tracked via very high frequency (VHF) transmitters annually since 2003 (Endriss et al., 2007; Wolf et al., 2013). Prior to the initiation of this grant, we completed intensive field surveys of TAFB to locate horned lizards between May and August 2019 using methodologies mirroring Endriss et al. (2007) and Wolf et al. (2013). The GPS locations and weight of all adult female lizards were recorded, and these individuals were tracked daily to monitor potential nesting behavior (females dropping significant weight over a single day were believed to have deposited eggs). We excavated areas surrounding the last known GPS location for these females and recovered three clutches of eggs in 2019. These clutches were transported to the OKC Zoo's Lizard Lab for artificial incubation and 15 hatchlings that survived from these clutches became the first cohort of adult Texas Horned Lizards that were released in 2021 at two years of age. We repeated the intensive field surveys described above during the summer of 2020 and located three additional clutches of eggs from which we reared 19 hatchlings that became the first cohort of juvenile lizards that were released in 2021. The collection of eggs in 2019 and in 2020, as well as the initial rearing of hatchling and juvenile horned lizards between August 2019 and September 2020, were completed prior to the initiation of this grant. However, beginning in October 2020, the rearing of young lizards was conducted under this grant. During the summer of 2021, two clutches of eggs were collected from TAFB and these resulted in 20 hatchlings that were reared for release for release as adults in 2023. In 2022, no gravid females or egg clutches were found on TAFB and we were unable to rear a cohort of juveniles for release that year. In 2023, we did not observe any gravid females on TAFB during June and July, however, we found multiple sets of hatchlings in July and August. Based on the locations of the hatchlings that were encountered, it appears that at least three adult females in the Tinker AFB population laid clutches in 2023. Nine hatchlings were found and brought to the zoo, to form a juvenile cohort that was released in May 2024 to help determine if soft releasing horned lizards as juveniles (rather than adults) is a more appropriate headstart strategy.

Animal Rearing:

Texas Horned Lizards at the Oklahoma City Zoo's *Lizard Lab* were housed individually in ~75 L glass terrariums (~76.20 × 33.02 × 33.02 cm) with approximately two inches of substrate consisting of a mixture of sand, mulch, decomposed granite, and soil. The aquaria were decorated with rock structures and cholla wood for refuge and basking. Each tank maintained a temperature gradient using an overhead 100 W basking bulb (Fluker's, Port Allen, LA, USA) for 8:16 hour on/off cycles on one side. Ultraviolet light was provided by a 10.0 UVB bulb (T5 HO ReptiSun, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA) spanning the length of the tank for 11:13 hour on/off cycles. We supplied water twice a day by misting the enclosure walls and artificial plants using a reverse osmosis and deionization filtered water setup with a misting system (MistKing, Jungle Hobbies Ltd., Emeryville, ON, Canada). Ambient temperature of the *Lizard Lab* remained at 28.6 °C and environmental conditions were not adjusted to replicate natural seasonal shifts that trigger brumation in wild Texas Horned Lizards. Due to the difficulty of acquiring harvester ants, the preferred prey of horned lizards, we fed the lizards a diet of fruit flies (*Drosophila hydei* primarily, some *D. melanogaster*) and pinhead crickets (*Acheta domesticus*) every other day, until they reached larger sizes at which time their feeding was increased to daily feedings that were a combination of fruit flies, crickets, and mealworms (*Tenebrio molitor*). Before feeding, all insects were dusted with a Repashy Calcium Plus supplement and/or Repashy Formic-Cal Plus AntEater supplement (Repashy Ventures Inc., Item #RSCP3). The prey items fed to lizards were primarily sourced from the online retailer Josh's Frogs, or OKC Zoo stocks (Josh's Frogs, LCC).

Each individual was weighed and measured biweekly to monitor growth and animal health (Figure 1). Ambient temperatures in the Lizard Lab were maintained above 70° F year-round with basking spots of ~100° F available to each lizard within their enclosures. Fecal samples from each lizard were collected when possible, to monitor gut microbial communities in individuals as the lizards age and eventually after animals are released.

As reported in the Interim Reports, we made adjustments to the feeding and watering protocols each year in the *Lizard Lab* to maximize animal growth and survival. These changes resulted in a protocol that appears well-suited for horned lizards in human care as survival rates improved substantially year over year. Only 49% of lizards hatched or collected in 2019 survived past their first year of life whereas 73% of animals from the 2020 cohort did. We achieved an 87% survival rate for lizards in the 2021 cohort and an 89% survival rate for the lizards in the 2023 cohort. The greatest rates of mortality of hatchlings occurred during their first four months (August through November). One of the husbandry improvements that we made in 2021 and continued through the life of the project was to soak the captive horned lizards in a shallow pan of water biweekly to prevent dehydration and weight loss (see protocol in Appendix A).

The Texas Horned Lizard cohorts that we reared were as follows:

2019-hatched: Thirty-two hatchlings were produced from three clutches of eggs that were collected on TAFB. Prior to the initiation of this grant, there were 17 mortalities between August 2019 and August 2020. However, there were no mortalities between the start of this grant in October 2020 and the release of this cohort back on to TAFB. The fifteen surviving individuals were released as adults in June of 2021 and were the first cohort to be released as adults.

2020-hatched: Twenty-six hatchlings were produced from three complete or partial egg clutches. Seven of these hatchlings died between August and November, but there were no further mortalities before their release. Nineteen individuals were released as juveniles in June of 2021 and were the first cohort to be released as juveniles.

2021-hatched: Thirteen hatchlings were produced from a single clutch of 16 eggs that were laid in the *Lizard Lab* by a gravid female from TAFB that was temporarily maintained there. In addition to these 13 hatchlings, eight hatchlings were captured in August 2021 on TAFB and moved to the *Lizard Lab*. Based upon their locations on TAFB, these eight hatchlings were believed to be the product of three different egg clutches. With an initial starting size of 21, there were three mortalities between October 2021 and February 2022. A fourth individual became seriously ill in July 2022 and was euthanized. In November 2022, another individual died unexpected of sand impaction in its digestive tract. The remaining 16 (nine males and seven females) were released as adults on to TAFB in May of 2023. This was the second cohort of headstarted adults.

2022-hatched: No egg clutches or hatchlings were found on TAFB and no cohort was reared for 2022.

2023-hatched: No gravid females were observed on Tinker AFB during June and July; however, multiple sets of hatchlings were found in July and August. Based on their locations, these hatchlings appeared to be the offspring from at least three females that laid clutches in 2023. Nine hatchlings were captured and brought to the *Lizard Lab* to form the 2023 cohort. One hatchling died in September 2023, but the remaining eight survived and were released on to TAFB as juveniles in May 2024. This was the second cohort of headstarted juveniles.

Following their release, each time that a headstart lizard was encountered, we collected cloacal swabs and/or opportunistic fecal samples to assess the gut microbial communities in these animals. These samples were compared directly to samples taken from the same headstart lizards while in human care at the *Lizard Lab* to improve our understanding of gut microbial community response to host reintroduction. We also sampled individuals from the resident population at TAFB seasonally to establish a baseline "wild" microbiome for comparisons with those observed in headstart lizards. Finally, we attempted to collect non-lethal blood samples from all wild and captive headstart animals for population genetic analysis; however, we experienced low success rates in successful blood draws from lizards. All microbiome samples were flash frozen in liquid nitrogen and stored at -20º C until processed in the lab.

Headstart Program Establishment:

Tracking and survivorship data from headstart lizards on TAFB are essential components of the Texas Horned Lizard headstart program at the Oklahoma City Zoo. Husbandry practices are equally important, and a Standard Operating Procedure was developed as a baseline guide for caring for captive Texas Horned Lizards in the *Lizard Lab* (Appendix A). Through continued monitoring of the Texas Horned Lizards that were released during the project's duration, we were able to assess: 1) the viability of headstart lizards on TAFB post-release and their capacity to reach reproductive status, 2) survival rates for Horned Lizards released at varying sizes/age classes, and 3) potential shifts in gut microbial ecology associated with host release. This information can be used to shape future release plans of Texas Horned Lizards onto TAFB and even potential releases of lizards across the state to supplement dwindling populations or reintroduce this iconic species to areas where populations have been extirpated locally.

TEXAS HORNED LIZARD RELEASE & MOVEMENT, HOME RANGE, AND SURVIVORSHIP TRACKING (OBJECTIVE 2)

Study Area:

Tinker Air Force Base (TAFB) is a 2,000-ha urban military installation located in Midwest City, Oklahoma, USA. Within the base lies our study site, Wildlife Reserve 3 (WR3), a multi-use 15-ha grassland composed of mixed native and non-native plant species, intersected with a loop walking trail, 2 man-made fishing ponds, and a small, wooded stand in the northwest quadrant. Further description of plant species and climate of WR3 can be found in Vesy et al. (2021). The reserve is flanked by residential housing to the north and west and military infrastructure to the south and east. Due to these surroundings, the residing population of Texas Horned Lizards is expected to be highly insular, with minimal immigration or emigration (Wolf et al. 2013, Wall et al. 2024). Numerous native species capable of depredating horned lizards also inhabit the reserve, including Western Ratsnakes (*Pantherophis obsoletus*), Speckled Kingsnakes (*Lampropeltis holbrooki*), Prairie Kingsnakes (*Lampropeltis calligaster*), Raccoons (*Procyon lotor*), Striped Skunks (*Mephitis mephitis*), Coyotes (*Canis latrans*), Bobcats (*Lynx rufus*), Opossums (*Didelphis virginiana*), and numerous birds of prey (Bogosian et al. 2012).

Headstarting, Soft-Release and Tracking Protocols:

Our study aimed to release both age classes (juvenile and adult) of headstarted lizards simultaneously for comparison with the resident population, however due to a lack of available hatchlings in 2022 to captive-raise to juvenile age, the study was staggered into three releases: a simultaneous release of an adult and a juvenile cohort in 2021, a release of an adult cohort in 2023 and the release of a juvenile cohort in 2024. At TAFB, individuals are considered to be hatchlings until they emerge from their first brumation at approximately seven to eight months of age, then as juveniles until they emerge from their second brumation as adults at roughly 20 months (Endriss et al. 2007). The first release was conducted in June of 2021 and consisted of 15 adults (hatched in 2019) and 19 juveniles (hatched in 2020).

These lizards were placed into four soft-release pens on Tinker Air Force Base's Wildlife Reserve #3 (WR3) on 1 June 2021. Substantial and unexpected mortalities occurred among the lizards while they were in the soft-release pens, and 14 deaths occurred over five weeks. It appears that a five-week soft-release duration is unnecessarily long for lizard acclimation because lizards were observed exhibiting natural behaviors immediately upon placement in soft-release pens and one lizard was even seen consuming native ant species right as it was placed in its pen for the first time. We recorded significant lizard die-offs after the third and fourth weeks of acclimation in the pens and it appears that some lizards did not find adequate shelter from increasing ambient temperatures in the limited confines of the soft-release pens. Because of this, we reduced the acclimation period during the subsequent releases of headstarted lizards and shifted the release time from early June to mid-May to avoid extreme heat and potential desiccation. In 2021, desiccation appeared to be the leading cause of mortalities for the headstart as well as the wild juvenile lizards during the latter half of June when period of unseasonably hot and dry weather occurred. On 8 July 2021, the 20 surviving headstart lizards from these release pens were affixed with solar-powered CTT LifeTags and harmonic radar diodes (placed on the 2019 sub-adult lizards) or with harmonic radar diodes only (placed on the 2020 juvenile lizards) to track these individuals weekly after their full release on WR3.

Lizards shed or removed their tracking tags/diodes with surprising efficiency over the summer making it difficult to assess survivorship and growth for the released individuals. From the 20 animals released completely in July, we relocated 18 individuals throughout the remainder of the first month. Four of those individuals were deceased when located, leaving at most 16 headstart individuals alive for the remainder of the season. Throughout August, we found only eight of these individuals (most of these were found more than once though). Similarly, only six individuals were encountered in September and just three in October, although many wild lizards also were exhibiting brumation behaviors and avoiding relocation in October. No mortalities were confirmed after the four that were lost in June, suggesting that as many as 16 lizards may still be alive on WR3 although most had shed their tracking devices. Furthermore, of the three lizards identified in October, we located two that had buried themselves in preparation for winter brumation. These lizards had been kept at stable temperatures and light cycles year-round in the *Lizard Lab* the winter before so we did not know if the lizards would emulate this critical behavior to survive the cold winters of Oklahoma. Because lizards were so capable of shedding or removing tracking technologies during this first headstart iteration, it is difficult to compare growth rates between cohorts statistically. Lessons learned from this first successful release of Texas Horned Lizards were used to improve the survivorship and tracking data in the subsequent releases in 2023 and 2024 and allowed for better comparisons of growth rates and survivorship to their wild counterparts.

Based upon our tracking and captures at TAFB during the summer and fall of 2021, we knew that at least two individual horned lizards had entered brumation and we expected that up to 16 individuals from the 2021 release might be alive at the start of the spring 2022 field season, although many of them had shed their transmitters and could not be tracked directly. Through 2022, however, we only found three of the potentially 16 individuals, one of which did not survive brumation. This meant that we only had two trackable headstarted lizards at the beginning of the season. We tracked one individual until 6/16/22, when her CTT signal stopped transmitting and we were unable to locate her again. The other individual was found deceased on 6/26/22 and showed no signs of predation. At the end of the season, we did not have any trackable released headstarted lizards in spite of repeatedly walking transects to search for horned lizards in all areas of Wildlife Reserve 3, including the area surrounding the headstart pens. Because of the unexpectedly low apparently survival rates of the 2019-hatched and 2020-hatched cohorts, these individuals were not included in our final analyses and instead we restricted our analyses of survival and dispersal on the 2021 cohort (released as adults in 2023) and the 2023 cohort (released as juveniles in 2024).

The second release took place in May of 2023 and consisted of 16 adults that were previously captured from Wildlife Reserve #3 in August 2021 as either as hatchlings or eggs and transported to the Oklahoma City Zoo to be captive-raised until adulthood. On 5 May 2023, we released 16 adult headstarted lizards (9 males and 7 females) after ensuring there would not be any inclement weather during their first three consecutive days in the soft release pen. The construction of the pen took place over the preceding three days after we first identified suitable habitat within the area occupied by the resident lizard population that was also far enough away from the loop trail to prevent anthropogenic disturbance. We then buried tan 36 cm vinyl flashing approximately 13 cm into the soil to form an 8 m by 9 m rectangle. The walls of the pen were reinforced by screwing the flashing to 60 cm garden stakes buried at 1 m intervals along the perimeter, and holes were drilled into the flashing to allow invertebrates to enter the pen. Additionally, we installed 10, 1.5 m T-posts within the pen that were connected with bailing wire to support a canopy of deer fencing attached to the buried garden stakes to prevent predator access. We weighed the lizards weekly to ensure that they were eating and that there was an adequate supply of prey available. After three weeks, the animals were fully released to the rest of the reserve after we removed the flashing from the corners of the pen. The timing of the release, along with the soft-release duration time, was chosen to avoid drawbacks from the previous releases in 2021 that was characterized by high mortality rates during a 5-week soft-release in the month of June.

To provide a basis for comparison, we also tracked a subset of similar-aged wild lizards on the reserve. The wild, resident adult lizards were found in early to mid-April of the 2023 active season, by relocating 13 lizards that had been previously marked and tracked at TAFB during 2021 and 2022. We captured six additional adults either through opportunistic encounters while tracking the tagged individuals, or during one of three inventory surveys that took place throughout the active season. These inventory surveys took place once per month from June through August, during which groups of researchers spent four days walking transects spaced 3 m apart throughout the reserve looking for new lizards. Upon capture for resident lizards, or within the week prior to soft release for the headstarted individuals, we implanted unmarked animals with an 8 mm passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) and secondarily marked them with a toe-clip on the fourth toe following the procedures previously used at TAFB (Vesy et al. 2021). We dorsally outfitted the lizards with 0.95 – 1.1 g very high frequency (VHF) radio transmitters (BD-2 series, Holohil Systems, Carp, Ontario, Canada), using 100% non-toxic silicone adhesive and collars made from 3 mm elastic bands sewn to the transmitter. Towards the end of the season, some resident lizards were instead outfitted with digital radio tags 1.0 g (HybridTag, Cellular Tracking Technology, Rio Grande, NJ, USA), which included a brown-painted, flexible diode (0.03 g, on average), glued on to the underside of the tag. To ensure the transmitters weighed less than 10% of each individual's body mass, we weighed and measured snout-vent length of each lizard before attaching it. VHF radio transmitters were replaced as needed before battery failure, and the digital radio transmitters were similarly replaced if they stopped functioning.

Once radiomarked, we used telemetry to home to the lizards' locations three to six times per week until the end of the active season in October, using either a TR-8 handheld receiver with Yagi antenna (Telonics Inc., Mesa, AZ, USA) or the CTT Locator (Cellular Tracking Technology) for VHF and CTT tags, respectively. If a lizard's tag appeared to be malfunctioning, we attempted to locate the animal for tag replacement by walking transects around its last known location while waving a harmonic radar receiver (Handheld model R9, RECCO Rescue Systems, Lidingo, Sweden), which emitted an audible ping when near a diode or VHF tag. Upon visual relocation, we recorded the lizard's Universal Transverse Mercator (UTM) coordinates (North American Datum 1983) using a handheld Trimble GeoExplorer computer (Trimble GeoXT, Terrasync 2.3, Strategic Consulting International, Oklahoma City, OK, USA), along with their survival status, whether they were active or burrowed, and any observed reproductive behavior. To minimize stress for the animals, we limited handling time by taking morphometric measurements during tag changes, with additional field measurements taken only periodically to monitor the lizards' growth and health.

For the third release, we had collected nine hatchlings opportunistically during August of 2023 and brought them to the OKC Zoo to be headstarted until their release as juveniles in May 2024. From this cohort, eight survived to be headstarted and soft-released using the same methodology as the adult cohort in 2023; however, they were fully released from the pen after two weeks (May 5th – 20th) when we noticed a slight decline in mass in a few individuals. To provide a basis for comparison, resident juveniles were captured between mid-May and mid-June in 2024 by opportunistic encounters while researchers continued tracking surviving adult lizards. Due to the juveniles' small mass (< 5 g), we opted to forgo using PIT tags to reduce potential stress on the animals and instead marked them with a unique combination of two toe-clips (Vesy et al. 2021). All juveniles were tagged with either a thin, flexible, diode if their weight was < 5 g, a 0.45 g VHF transmitter (BD-2x series, Holohil Systems) if they weighed between 5 and 10 g, or 0.95 – 1.1 g BD-2 VHF transmitters if they exceeded 10 g. Tag changes, morphometric measurements, and population tracking methods remained the same for the juveniles, and the surviving adult lizards, until the end of the study in October 2024.

Analysis of Movement and Home Range:

To compare movement behavior between headstarted and resident groups, we estimated daily movement rates using the *calculate motion statistics* tool in ArcGIS Pro (ESRI, Redlands, CA, USA) to measure the geodetic distance between individuals' consecutive fixes, then we divided that value by the number of days that had elapsed. Since this method likely underestimates the actual distances moved, we minimized this bias by only including fixes where no more than two days had elapsed since the previous one (i.e. one day in between fixes where the lizard was not located). For accurate group comparisons, we calculated daily movement rates for residents by only using relocations collected after the release of the headstarted group. We also only used data collected during the 2023 active season to analyze adult movement and home range due to sample size limitations by the 2024 season.

To account for the repeated measures per individual, the varying intervals in which different individuals remained in the study, and the right skew of the data, we analyzed natural log-transformed daily movement rates using linear mixed models (LMMs). To do this we used the *lme4* package (Bates et al. 2015), and all subsequent statistical analyses where conducted in R v4.2.2 (R Core Team 2022). We fit models with the predictor variables *origin* (whether the lizard belonged to the headstarted or resident group) and *days* (number of days since the onset of the study period) as fixed effects, and *individual lizard ID* was treated as a random effect. The *days* variable was first centered and scaled (total # of days per season ~ 120, mean = 0, SD = 1) before inclusion in the models for model stability and computational efficiency. We used Akaike Information Criterion (AIC) model selection to compare a series of models fitted using restricted maximum likelihood (REML), all of which included an interaction between the variables (*origin* * *days*), but varied in their random effect structure: random intercept-only, random slope-only, or random intercept-and-slope. After ensuring that the model with the lowest AIC met model assumptions, we included its random effect structure in a set of models fitted using maximum likelihood that varied in their fixed effects: null, *origin* only, *days* only, additive effects (*origin* + *days*), and a full interaction (*origin* * *days*). Finally, the most parsimonious model was refit using REML to estimate parameters and we used the *MuMIN* package to calculate the conditional and marginal coefficient of determination values (Bartoń 2024). The conditional R^2 measures the variance explained by both the random and fixed effects, while the marginal R^2 measures the variance explained by the fixed effects only.

Following the framework put forth by Signer et al. (2019), we compared the home ranges of headstarted and resident lizards using multiple estimators, despite our expectation that the relative comparison between groups would be robust to estimator choice. Limitations in our tracking methodology and subsequent data did not allow for the use of modern estimation techniques that incorporate animal movement into model design; however, our movement data is available on Movebank to facilitate inter-study comparisons (Crane et al. 2021, Silva et al. 2022). For individuals with greater than 10 relocations, we estimated their home range area in hectares derived by 95% minimum convex polygons, or hulls formed by the 95% isopleths generated by kernel density estimation (KDE) using the *AMT* package (Signer et al. 2019). The KDEs derived using the reference bandwidth selection method (h_{ref}) were over-smoothed, leading to area overestimates, while the least-squares cross-validation (*lscv*) method often failed to converge. Instead, we present estimates obtained using plug-in bandwidth selection with a spatial resolution of 2 m, though we acknowledge this method resulted in under-smoothed utilization distributions for some individuals (Pagano et al. 2021). We also noted that increasing the spatial resolution did not meaningfully change the relative differences between headstarted and resident groups. Because home range estimates can be underestimated for animals with few relocations, we performed preliminary Spearman's correlation tests to evaluate if area estimates correlated with the number of relocations for each estimator. Statistically significant results were not observed for any of the tests at an alpha level of 0.05. We performed Student's t-tests to compare the mean area used between the headstarted and resident groups for both estimators, after first applying a natural logarithm transformation to meet test assumptions. Finally, we measured the furthest distance any headstarted individual moved from the release site and summarized this information for both age-classes.

Analysis of Survival and Body Condition:

We used the *survival* package to compare lizard survivorship between the headstarted and resident groups for both age classes (Therneau 2024). Survival rates were obtained using the Kaplan-Meier estimator, as this method allows for right censoring and delayed entry of individuals (Pollock et al. 1989). Since resident lizards could enter the study a few weeks before or after the headstarted lizard release, we applied a risk-set adjustment to include an individual in the risk-set at each mortality event only if they had been previously located. This method prevents bias in survival probabilities that can arise from left truncation, but relies on the assumption of "independent delayed entry", that is, an individual's hazard risk is independent of entry time (Backenroth et al. 2022).

An animal was considered deceased if we found their carcass, we found their transmitter visibly damaged or with no sign of shedding, or if we located the predator (in this case snakes) that had consumed them. Nearly all the transmitters or the remains thereof were recovered within five days of the animal's last relocation. Mortality was dated as the day an individual's tag or remains were found if their last fix was the day prior, or if multiple days had passed, as a date approximately in the middle of the time interval between their last fix and recovery of their body or tag. Alternatively, an animal was coded as right-censored on the date their transmitter failed to produce a traceable signal, their shed tag was recovered, or the day they left the reserve and could no longer be tracked.

Survival analysis spanned from April 2023 through October 2024 for the adult age class and throughout the 2024 active season for the juveniles (May to October). Unfortunately, one headstarted juvenile had to be euthanized due to a broken jaw and we were unable to determine if the injury occurred during predation escape or if they were accidentally injured the day prior by volunteers or wildlife reserve visitors. Thus, we report a high estimate of survival for the headstarted juvenile cohort by treating that individual as censored, and a low estimate considering them to be predated. To be conservative in our survival comparison we treated that individual as deceased.

For both age classes, we constructed Cox proportional hazards models containing the predictor variable *origin* to obtain the hazard ratio between headstarted and resident groups. A second model included *entry date* (the number of days since the onset of the study when an individual was included in analysis) as an additional covariate (Cox 1972). To test the independent delayed entry assumption, a likelihood ratio test was used to compare the goodness of fit of both models. If the null hypothesis is not rejected there is evidence that the assumption was met (Backenroth et al. 2022). Before exploring outputs from all constructed Cox proportional hazard models, we first ensured that the proportional hazards assumption was met (Therneau 2024). Given the small sample of juveniles, in addition to construction of a Cox proportional hazards model, we further evaluated the differences in survival rates by calculating 95% confidence intervals around the mean difference in survival between the 2 groups on day 130, the last day of the season, using 10,000 bootstrap resamples.

We used each lizard's mass as an indicator of its body condition to determine if it was adequately foraging and maintaining weight in the wild. Before summarizing mean mass measurements for the different age classes and groups, we subtracted the average weight of the transmitter if an individual was wearing it during measurement. We pooled both sexes of headstarted adults that had two or more mass measurements, spaced at least a month apart, and performed a two-sided paired t-test between their pre-release weight and their last active season measurement. When calculating the mean mass for residents, we used their maximum measurement and if the individual was female, we did not include their measurements if they were likely gravid (Endriss et al. 2007). Juveniles were expected to have substantial increases in body size throughout the season, so both headstarted and resident lizards' weights were summarized from June through August by averaging individuals' measurements across the month. There were too few resident juveniles in May, so for comparative purposes, measurements taken towards the end of May were binned together with June measurements for both groups.

CHARACTERIZING GUT MICROBIAL COMMUNITIES (OBJECTIVE 3)

Microbial Community Sampling:

To assess the contributions of diet and environment on headstarted Texas Horned Lizard microbiomes during transitions from captivity to wild release, we collected samples of the headstart lizard gut microbiome, and microbial communities associated with their diet and environment from January to July 2023. For a baseline comparison with newly released headstarted lizards, we also sampled the gut microbiome of the wild, resident population on Wildlife Reserve 3 from May to July 2023. All sample types (dietary microbiome, environmental microbiome, and gut microbiome) were collected once per month. We wore sterile gloves during sample collection and immediately placed all samples into a 1.5 mL cryogenic vial with Zymo DNA/RNA Shield (Zymo Research Products, Item #R1100) and stored them at -20°C until DNA extraction.

Gut Microbiome Sampling:

To evaluate the headstart lizard gut microbiomes, we collected gut microbiome samples from all 16 headstart lizards twice per month. From May to July 2023, we additionally sampled the gut microbiome of all tracked resident lizards once a month. Each month, approximately nine to twelve lizards were tracked; the total number of tracked lizards fluctuated slightly per month with lizard mortalities and VHF tag failures leading to censored individuals. Gut microbiome samples were collected by gently inserting a sterile swab (Puritan Medical Products, REF #25-1000 1PD) into the cloaca and rotating it 3–4 times. It has been demonstrated that cloacal swabs are an effective method to sample the gut microbiomes of lizards (Smith et al., 2021; Bunker et al., 2022). We extracted total microbial DNA from 144 headstart lizard gut microbiome samples and 26 resident lizard gut microbiome samples.

Environmental Sampling:

To assess the contribution of the environmental microbiome to the headstart lizard gut microbiome, from January to April 2023, we collected 10 swab samples of the microbiota present in the headstart lizard's enclosures each month. We used a random number generator to randomly select 10 enclosures to sample. While the lizards were in captivity, we sampled both the enclosure substrate (a mixture of sand, mulch, decomposed granite, and soil) and water from the MistKing misting system in each enclosure. Once the headstart lizards were translocated to Wildlife Reserve 3 at TAFB, we collected soil microbiome samples in the locations where headstart lizards were found from May to July 2023. We collected at least 10 soil samples per month. To sample microbiota present in the lizards' environment, we moved a sterile swab across the surface of the cage substrate or soil for 5–10 seconds. To sample water from the enclosure misting system, we inserted a sterile swab into the stream of mist and rotated it for 5–

10 seconds until the swab was well-saturated with water. We extracted total microbial DNA from 56 environmental samples.

Dietary Sampling:

To assess the contribution of dietary microbiota to the headstart lizard gut microbiome, we sampled microbial communities associated with prey items fed to the lizards in captivity and potential prey items present on Wildlife Reserve 3 of TAFB. From January to April 2023, while the lizards were housed in captivity, we collected 10 samples of each prey items that were fed to the lizards. We also collected 10 samples of the Repashy Calcium Plus supplement and/or Repashy Formic-Cal Plus Ant Easter (Item #RSCP3). Lizards were fed a combination of insects including fruit flies (*Drosophila hydei* primarily, plus some *D. melanogaster*), crickets (*Acheta domesticus*), and mealworms (*Tenebrio molitor*) dusted with Repashy Calcium Plus supplement and/or Repashy Formic-Cal Plus Ant Eater (Item #RSCP3) once per day. Whole insects were collected wearing sterile gloves. We sampled the Repashy Calcium Plus supplement and/or Repashy Formic-Cal Plus Ant Eater supplement (Repashy Ventures Inc., Item #RSCP3) by introducing a sterile swab into the supplement bottle and swirling it throughout the bottle for 3–4 seconds.

After the headstarted lizards were released, four insect pitfall traps were placed in areas of high lizard activity to capture the diversity of possible prey items at Wildlife Reserve 3. Texas Horned Lizards are generally considered harvester ant (*Pogonomyrmex* spp.) specialists, but there are currently no known populations of harvester ant on the Reserve (Ramakrishnan et al., 2018). Instead, resident lizards have been shown to consume other ant genera such as *Pheidole* and *Formica* (Ramakrishnan et al., 2018). Additionally, other wild populations of Texas Horned Lizards have been shown to depredate other non-ant arthropods (Heuring et al., 2019; Richards and Watson, 2024). To sample the total possible diversity, we used all arthropods present in traps. The traps consisted of small plastic cups placed approximately 4 inches deep into the ground and filled to one inch with a mixture of dish soap and water. Insect traps were checked once or twice a week approximately 48–72 hours after setting. If a lizard was located near a likely prey item, or observed eating, we also opportunistically collected those prey items. Prey items were collected with sterile forceps, and then grouped based upon order including *Coleoptera* (beetles), *Araneae* (spiders), *Orthoptera* (grasshoppers/crickets), and *Diptera* (flies), *Hymenoptera* (ants), and then washed with ethanol. We extracted total microbial DNA from 49 diet samples.

DNA Extractions:

We extracted total microbial DNA from 170 lizard gut microbiome samples, 56 environmental samples, and 49 diet samples using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Products, Item #D4300T). For each DNA extraction batch, we included a blank sample to serve as a negative control. We also included two microbial community standard samples (Zymo Research Products, Item #D6300) in an extraction batch to serve as a positive control.

All swab samples (gut microbiome and environmental samples) were extracted following kit protocols. Additional steps were taken to extract whole insect samples. First, individual insect specimens were removed from DNA/RNA shield and cut into small pieces using a sterile razor blade on a sterile surface (Watson et al., 2023). Each insect sample was then lysed twice, first using a lysis tube with larger 0.1 and 2.0 mm beads (Zymo Research Products, Item #S6014) to ensure the insect tissue was completely homogenized before being transferred into the standard 0.5 and 0.1 mm bead lysis tube (Zymo Research Products, Item #S6012) to disrupt the microbial cell walls (Watson et al., 2023).

Library Preparation:

After extraction, the DNA concentration in each sample was quantified using a Qubit Fluorimeter (ThermoFisher, Item #Q33238). No environmental samples of the captive lizards' water source contained detectable amounts of DNA and thus were excluded from the final sample set that was used to develop the metagenomic libraries. Following the protocol described in Kozich et al. (2013), we performed a one-step PCR to amplify the V4 region of the 16S ribosomal RNA (rRNA) gene. Included in the PCR reaction was a sample of a microbial community DNA standard as an additional positive control (Zymo Research Products, Item #D6305). The PCR product was cleaned with KAPA pure beads at a concentration of 0.6 to remove potential adapter or primer dimer (Roche Sequencing, Item #07983298001). Before completing the bead clean up protocol, all samples were normalized to 20 uL post-PCR with sterile laboratory grade water to ensure a consistent KAPA pure bead to sample ratio during the bead clean up protocol. The bead cleanup protocol was completed by an Agilent Bravo robot with a 96LT head at the Oklahoma Medical Research Foundation (OMRF) Consolidated Core Lab. Next, all bead-cleaned PCR products were quantified with a Qubit Fluorimeter, normalized to 10 nM of DNA, and pooled into a sterile, 1.5 mL micro-centrifuge tube. Pooled libraries were submitted to the OMRF Consolidated Core Lab for 2 x 250 base pair paired-end sequencing on an Illumina MiSeq machine.

Sequence Analysis:

Remnant adapter sequences were trimmed using AdapterRemoval v2 (Schubert et al., 2016). Sequence data were processed using the QIIME2 microbiome software package (Bolyen et al., 2019). We performed closed reference clustering with a similarity threshold of 99% against the SILVA 138.1 database (Quast et al., 2013) using VSEARCH (Rognes et al., 2016). From our initial dataset consisting of 286 samples, we obtained a total of 2,891,785 sequences that clustered into 32,777 operational taxonomic units (OTUs). First, we filtered out any archaea or nonbacterial sequences from the OTU table (Eliades et al., 2021). We additionally filtered out OTUs that were not present in at least two samples; assuming these features are likely PCR or sequencing errors if only present in one sample. Our dataset was then rarefied to a minimum sequence count of 500 for downstream analysis based upon the Shannon Diversity Index and observed OTU rarefaction curves (Supplementary Figures S2, S3). Out of the original dataset, one diet sample and one environment sample were excluded due to poor sequence quality. Some lizards had duplicate gut microbiome samples within one sampling period (biological replicates); we retained only the sample with the greater number of sequences. After all filtering, 214 samples were used for downstream analysis. Raw sequence data from this study can be found in the Sequence Read Archive (SRA) under BioProject PRJNA1218705.

Statistical Analysis:

To evaluate changes in the gut microbiome of headstart lizards, resident lizards, and microbial communities associated with their diet and environment, alpha diversity (within sample) and beta diversity (among sample) analyses were performed using QIIME 2 (Bolyen et al., 2019). Alpha diversity (Shannon Diversity, OTU Richness) and beta diversity (Unweighted

UniFrac Distance, Weighted UniFrac Distance) metrics were calculated for each sample using QIIME 2 (Bolyen et al., 2019).

First, we examined the variation in Shannon Diversity and OTU Richness of the headstart lizard gut microbiome over time. We built linear mixed models in R with the alpha diversity metric (either Shannon Diversity or Observed OTU) as the response variable, month of sample collection as a categorical fixed effect, and individual lizard identification as a random effect variable to account for individual variation. All linear models were run with restricted maximum-likelihood estimation using the package lmerTest (Kuznetsova et al., 2017). We checked model assumptions with QQ plots and performed statistical analysis using the Anova function in the statistics package (Chambers and Hastie, 1992). Finally, we used the package Emmeans to obtain pairwise comparisons from our models while adjusting significance for multiple testing with the Tukey method (Lenth et al., 2024).

Further alpha diversity comparisons were completed using the Q2 diversity plugin available through the QIIME2 software, which completes pairwise Kruskal-Wallis tests to determine group significance (Kruskal & Wallis, 1952; Bolyen et al., 2019). Each set of analyses was repeated for Shannon Diversity and OTU Richness. First, we separately compared the alpha diversity levels of the gut microbiomes of post-release headstart lizards and resident lizards less than one month after the release in May 2023, and two months post-release in July 2023. Due to the low number of resident lizards post-rarefaction (n = 3), we could not complete an alpha diversity comparison of June headstart and resident gut microbiomes. Next, we compared the alpha diversity of microbial communities present in the Texas Horned Lizard environment and diet across captivity and the wild. For all alpha diversity analyses, we considered a p-value <0.05 to be statistically significant.

To assess compositional differences among microbial communities, we utilized two beta diversity indices: Unweighted UniFrac and Weighted UniFrac distances. Both indices consider phylogenetic distances between observed OTUs, but the Weighted UniFrac distance also considers abundance, whereas Unweighted UniFrac distance is calculated based upon the presence or absence of OTUs (Knight et al., 2018). All beta diversity comparisons were completed using the Q2 diversity plugin available through the QIIME2 software, which completes pairwise PERMANOVAs to determine group significance (Anderson, 2008; Bolyen et al., 2019). Each set of analyses was repeated using Unweighted UniFrac and then Weighted UniFrac distances. First, to replicate the structure of our alpha diversity analysis, we separately compared the beta diversity of headstart and resident gut microbiome samples less than one month after the release in May 2023, and two months post-release in July 2023. Next, we completed another set of beta diversity analyses including all lizard gut microbiome, diet, and environment samples collected in both captivity and the wild. To best determine broad differences across these diverse microbial communities, we grouped the samples as follows: headstart gut microbiome pre-release, headstart gut microbiome post-release, resident gut microbiome, diet microbiome pre-release, diet post-release, environment microbiome pre-release, and environment microbiome post-release. We incorporated these beta diversity data into a principal coordinates analysis (PCoAs) using the QIIME2 software and then exported the results into the R environment for visualization with the package ggplot2 (Wickham, 2016).

To assess intra-individual changes in headstart lizard gut microbiomes, we identified the breadth of change (intra-individual Unweighted or Weighted UniFrac distance) in an headstart individual's gut microbiome on a monthly basis from January 2023 to July 2023. We hypothesized headstart individual's gut microbiome communities would display the greatest shift from April to May; reflecting the shift from a captive to wild environment. Although we aimed to extract and sequence at least two gut microbiome samples per individual per month, no individual lizard had a representative gut microbiome sample in every month from January to July 2023 due to poor sequence quality in some cases. Despite this limitation, we visualized intra-individual changes in headstart lizard gut microbiomes through volatility plots created using the Q2 longitudinal plugin available through the QIIME2 software (Bokulich et al., 2018; Bolyen et al., 2019).

POPULATION GENETICS OF TAFB HORNED LIZARDS (OBJECTIVE 4)

Population Genetic Analyses:

This objective was not completed, but in our original proposal we had planned to collect population genomic data from all frozen non-lethal genetic samples using a reduced-representation genomic approach called 3RAD, which can generate 100,000s of genomic markers for a comparatively low cost (Bayona-Vásquez et al., 2019). The 3RAD approach allows for a larger number of samples to be combined into a single sequencing lane (the most cost-prohibitive step). Data manipulation and organization would have employed iPyRRD (Eaton, 2014), prior to assessing key population genetic metrics (e.g., heterozygosity, inbreeding), and conducting analyses to assess population structure, including PCAs, STRUCTURE plots, and AMOVAs (similar to Lanier et al., 2015). Additionally, we planned to assess effective population size for the TAFB THL population using SNeP (Barbato et al., 2015). However, we were unable to complete the collection and analysis of population genetic data for the TAFB THL population as described during annual project reports. We experienced several unforeseen setbacks including delays caused by the global COVID pandemic and the unexpected departure of a trained and experienced graduate student who was capable of performing the procedures and analyses. We were unable to train ourselves or recruit an experienced student, so we stopped pursuing this objective in 2023 and redirected our resources toward the other objectives and in particular the collection and analysis of the gut microbial objective. We have continued to collaborate with Dr. Dean Williams at Texas Christian University (TCU), who also has an active conservation-focused horned lizard research program, and is engaged in a robust population genomic study of *P. cornutum* across a substantial part of the species' geographic range. As such, we can still determine what genetic populations the TAFB horned lizards are closely related to the dominate genotypes that have been documented in northern Texas and elsewhere in Oklahoma.

Summary of Progress:

Results

As mentioned earlier, our analyses for the movement, home range, and survivorship of the headstarted Texas Horned Lizards are based entirely upon the 2023 and 2024 releases of an adult and a juvenile cohort respectively. The initial headstart release that we conducted in 2021 was our first attempt and the lizards experienced high rates of tag losses and mortalities. We don't believe that the 2021 releases are directly comparable to the subsequent releases and we excluded them from our analyses.

Movement and Home Range:

Both age classes demonstrated relatively sedentary movement patterns (Table 1). We removed one headstarted adult and one resident juvenile as outliers from the spatial analysis for abnormally low movement rates compared to the other adult lizards and insufficient tracking data, respectively. Akaike information criterion (AIC) model selection for the random effects component of our candidate log-transformed daily movement rate linear mixed models (LMMs) indicated that the model including random intercept & slope effects was the best fit for both age classes; however, they differed in the most parsimonious model when selecting for fixed effects. The top model for adult movement included 1,066 observations and the additive fixed effects of *origin* and *days* (scaled). It accounted for 84% of the cumulative model weight, with a ΔAIC 3.5 units lower than the next best model. Both parameter estimates were statistically significant, providing evidence for higher movement rates in the resident adults ($\beta_{resident} = 0.978$, CI = 0.779 – 1.373), and gradually declining movement throughout the season ($\beta_{\mathrm{days}} = -0.328$, CI = -0.487 – -0.172). After accounting for individual and seasonal variation, a three-fold difference in daily movement rates between the two adult groups was detected on the linear scale. The fixed effects accounted for 55% of the conditional variance ($R^2 = 0.294$). In contrast, model selection for juvenile movement supported the null model as the most parsimonious. It included 586 observations and accounted for 55% of the cumulative model weight, with a ΔAIC 2.2 units lower than the next competing model. Therefore, we found no evidence that juvenile daily movements were influenced by their *origin* or the date of observation.

Home range estimates coincided with the movement trends observed for both age classes (Table 2; Figure 1). Headstarted adults had lower area estimates on average than residents for both 95% Multiple Comparisons Procedure (MCP) ($t_{28} = -3.925$, $P < 0.001$) and 95% Kernel Density Estimation (KDE) methods ($t_{28} = -3.769$, $P < 0.001$), and there was no evidence supporting a difference in area use between headstarted and resident juveniles for either the MCP ($t_{14} = -0.356$, $P = 0.727$), or KDE method ($t_{14} = 0.0646$, $P = 0.949$). Maximum dispersal distances from the release site for the adult headstarted cohort ranged from 29.78 – 142.02 m, with a median of 71.9 m (IQR = 53.43 – 114.04). The headstarted juveniles' maximum dispersal ranged from 13.87 – 292.93 m, with a median of 96.45 m (IQR = 56.09 – 199.64).

Survival and Body Condition:

All individuals from the headstart cohorts released in 2023 (adult) and 2024 (juvenile) survived the soft-release period; however, they had lower survival rates than wild lizards by the conclusion of the study (Table 3; Figure 2). For the adult age class, individuals that survived to enter brumation also survived throughout the wintering period, which ended within 10 days before or after April 1, 2024. Therefore, we consider survival to that date to be an approximate representation of each group's annual survival rate. We used the Cox proportional hazards models without the entry date covariate, as the likelihood ratio test was insignificant ($\chi^2 = 1.021$, $P = 0.312$). This model detected a nearly four-fold greater hazard risk for the headstarted lizards compared to the resident adults for the entire study period (HR = 3.63, CI = 1.38 – 9.59, $z = 2.606$, $P = 0.009$). In contrast, the Cox proportional hazards model for juvenile survival, which treated the ambiguous headstart death as a predation, did not detect a difference in hazard risk between the origin groups (HR = 3.165, CI = 0.850 – 11.80, $z = 1.303$, $P = 0.086$). Neither did the bootstrap analysis, as the confidence interval around the mean difference between the 2 groups' end of season survival rates (day 130) after 10,000 resamples included 0 ($\bar{x} = 0.327$, CI

= -0.193 – 0.778). However, we acknowledge that uncertainty around the HR is likely a reflection of our small sample sizes.

Before being released from the soft release pen, the 5 headstarted adult females had a mean weight of 13.44 g (± 1.05 SD), which increased to 15.44 g (± 1.78) for their end measurements. The 7 headstarted males, whose pre-release weight averaged 11.45 g (± 0.76), remained constant, ending with a mean of 11.40 g (± 0.78). We did not detect a statistically significant difference between average pre-release and post-release weights after pooling the sexes (t_{11} = -1.937, p = 0.079). Resident adult females reached larger body sizes than their headstarted counterparts on average ($\overline{x}$ = 18.35 ± 2.32 g, n = 8), as did the resident males ($\overline{x}$ = 15.21 ± 1.38 g, n = 8). The headstarted juvenile cohort's average mass for the pooled sexes increased from June ($\overline{x}$ = 11.18 ± 2.48 g, n = 8) to August ($\overline{x}$ = 13.37 ± 0.70 g, n = 3); resident juveniles, although much smaller on average in June ($\overline{x}$ = 6.07 ± 2.05 g, n = 9), reached similar sizes by August (13.07 ± 2.26 g, n = 6) (Figure 3). Note the discrepancy in sample size between juvenile groups in August.

Mortality and Reproduction:

Among both age classes, predation accounted for nearly all mortalities, however, we were only able to clearly identify Specked and Prairie Kingsnakes as predators (Supplementary Table S1, Figure S1). Interestingly, at least 9 of the headstarted adults survived 83 days post-release before a mass predation event by a small mammalian predator killed 5 individuals within 48 hours. These individuals, along with another lizard whose tag was recovered similarly damaged, were residing within 75 m of each other along the northern fence corridor separating the reserve from the mowed residential turf grass (Figure 1). Throughout 2024, we found the bodies of 2 headstarted and 3 resident adults, as well as 3 headstarted and 2 resident juveniles, all with their head, or portions of them, removed. We also observed two Hispid Cotton Rats (*Sigmodon hispidus)* that died in a similar manner. An avian predator may have been responsible for these unusual deaths; however, a previous study on predation of Texas Horned Lizards by Swainson's hawks (*Buteo swainsoni*) found 33 intact lizard heads in nests without their bodies, suggesting that either the heads were left over from dismemberment of the lizard, or the heads were removed at the nest to avoid injury (Lazcano et al. 2017). Given their small head size, sharp occipital and temporal horns, and tough dermal scales, it seems unlikely the heads were being preferentially targeted and consumed. The nature of these deaths, combined with multiple sightings on the reserve, points to cats (*Felis catus*) from the surrounding residential areas as the potential culprits.

In 2024, we observed reproductive behaviors in 2 of the surviving headstarted adults. In May, the male was observed attempting to copulate with an headstarted juvenile female, who had reached adult sizes due to headstarting. Then, in mid-June a female dropped approximately 7 g and had been digging a nest evidenced by the mud caked on her claws. The timing and amount of weight dropped, along with the nest digging behavior, coincided with the wild nesting females; however, we were unable to locate the exact nest location for confirmation.

Microbiome Analyses:

Our initial dataset consisted of 286 samples: 170 lizard gut microbiome samples, 49 prey item microbiome samples, 56 environmental microbiome samples, eight negative samples, two DNA microbial community standards and one PCR microbial community standard. From our initial dataset consisting of 286 samples, we obtained a total of 2,891,785 sequences that clustered into 32,777 OTUs with a similarity threshold of 99% against the SILVA 138.1 database (Quast et al., 2013). After filtering processes (including rarefying the dataset to a sequence count of 500), we retained 214 samples containing a total of 2,644,360 sequences that clustered into

21,366 OTUs (Quast et al., 2013; Supplementary Table S2). Raw sequence data from this study can be found in the Sequence Read Archive (SRA) under BioProject PRJNA1218705.

Taxonomic Composition of Microbial Community Samples:

While housed in captivity, prior to release, headstart lizard gut microbiomes were composed primarily of the phyla Proteobacteria (average relative abundance of 54.28%), followed by Firmicutes (18.89%), and Actinobacteriota (15.06%; Figure 4; Supplementary Table S3). Other dominant phyla (average relative abundance >1%; Suenami et al., 2019; Smith et al., 2023) included Bacteroidota (4.42%), and Chloroflexi (2.80%; Figure 4; Supplementary Table S3). After the headstart lizards were released to the wild, Actinobacteriota (32.63%) and Proteobacteria (29.88%) were the most abundant phyla (Figure 4; Supplementary Table S3). Other phyla present at an average relative abundance of between 1% and 10% included Firmicutes, Deinococcota, Planctomycetota, Myxococcota, Verrucomicrobiota, and Cyanobacteria (Figure 4; Supplementary Table S3).

Similarly to post-release headstart gut microbiomes, the phyla Actinobacteria (average relative abundance of 32.14%) and Proteobacteria (31.45%) were also the most abundant phyla in the wild resident gut microbiomes (Figure 4; Supplementary Table S3). Deinococcota (11.58%) and Firmicutes (9.27%) were also prominent phyla, while Acidobacteriota, Bacteroidota, Chloroflexi, Cyanobacteria, Planctomycetota, and Myxococcota were present at average relative abundances between 1–5% (Figure 4; Supplementary Table S3).

Microbial communities associated with the prey items fed to the headstart lizards in captivity consisted primarily of the phylum Firmicutes (average relative abundance of 41.13%), followed by Proteobacteria (35.56%), and Cyanobacteria (12.48%; Figure 4; Supplementary Table S3). Bacteroidota, Actinobacteriota, and Verrucomicrobiota were present at relative abundances between 1–10% (Figure 4; Supplementary Table S3). In the wild, the most abundant phyla associated with prey items was Proteobacteria (68.49%), followed by Cyanobacteria (11.70%). Other phyla present in average relative abundances from 1–10% included Bacteroidota, Firmicutes, Actinobacteriota, Myxococcota, and Acidobacteriota (Figure 4; Supplementary Table S3).

Microbiomes associated with the captive environment (i.e. headstart lizard enclosures) were dominated by Proteobacteria (30.70%), Firmicutes (18.67%), and Actinobacteriota (13.1%; Figure 4; Supplementary Table S3). The phyla Chloroflexi, Bacteroidota, Planctomycetota, Acidobacteriota, Verrucomicrobiota, Gemmatimonadota and Myxococcota were present at average relative abundances between 1–10% (Figure 4; Supplementary Table S3). Similarly to the captive environment, Proteobacteria (32.27%) was the most abundant phyla associated with wild environmental microbiomes. Actinobacteria (16.79%) and Acidobacteria (14.01%) also comprised large portions of the wild environmental microbiome, while Bacteroidota, Cyanobacteria, Planctomycetota, Myxococcota, Chloroflexi, Verrucomicrobiota, Firmicutes, and Gemmatimonadota were present in relative abundances from 1–10% (Figure 4; Supplementary Table S3).

Alpha and Beta Diversity Analysis:

From our linear mixed models, we found a significant effect of month of the year on Shannon Diversity (F(6, 73.88) = 11.482, $p < 0.001$; Supplementary Table S4) and OTU richness (F(6, 74.531) = 21.361, $p < 0.001$; Supplementary Table S5) of headstart lizard gut microbiomes.

While in captivity (January–April), the alpha diversity of headstart lizard gut microbiomes was relatively stable, and no significant differences were observed between sampling periods except for OTU richness in March and April (Supplementary Table S5). Across both metrics, May (<1 month post-release), appears to be a peak in diversity (Figure 5). The Shannon Diversity and OTU Richness of headstart lizard gut microbiomes in May were significantly different from all other months except June (Supplementary Tables S4, S5). Both metrics decreased in June, and then by July were no longer significantly different from any of the months in captivity (Figure 5, Supplementary Tables S4, S5).

We found significant differences between headstart and resident lizard gut microbiomes in May 2023 for both alpha diversity metrics evaluated: Shannon Diversity ($H = 14.84$; $p < 0.001$; Figure 5; Supplementary Table S6) and OTU Richness ($H = 12.71$; $p < 0.001$; Figure 5; Supplementary Table S6). When comparing headstart and resident lizard gut microbiomes in July 2023, two months post-release, no significant differences were observed for either diversity index: Shannon Diversity ($H = 2.04$; $p = 0.153$; Figure 5; Supplementary Table S6) and OTU Richness ($H = 0.003$; $p = 0.96$; Figure 5; Supplementary Table S6).

To assess differences in microbial community diversity between the Oklahoma City Zoo and TAFB, we compared the alpha diversity of microbial communities recovered from diet and environment samples collected in each location. Environment microbial community samples exhibited significant differences in Shannon Diversity ($H = 4.40$; $p<0.05$; Supplementary Table S6), but not OTU Richness ($H =0.09$, $p = 0.76$; Supplementary Table S6). Captive and wild diet microbial community samples did not differ in Shannon Diversity ($H = 0.23$; $p = 0.63$; Supplementary Table S6) or OTU Richness ($H = 1.07$; $p = 0.30$; Supplementary Table S6).

To replicate the structure of our alpha diversity comparisons, we compared beta diversity metrics for headstart and resident lizards in May and July 2023. We calculated and compared two beta diversity indices: Unweighted UniFrac distance and Weighted UniFrac distance. During the month of May, both Unweighted UniFrac analysis and Weighted UniFrac analysis resulted in significant differences ($p = 0.006$ and $p = 0.025$, respectively; Supplementary Table S7). During the month of July, neither analysis resulted in significant differences between headstart lizard and resident lizard gut microbiomes ($p = 0.176$ and $p = 0.107$, respectively; Supplementary Table S7).

We also analyzed beta diversity differences (again Unweighted UniFrac distance and Weighted UniFrac distance) across all sampled microbial communities (headstart lizard, resident lizard, diet, and environment). To best determine the compositional differences across these diverse microbial communities, we grouped samples as following: headstart gut microbiome pre-release, headstart gut microbiome post-release, resident gut microbiome, diet pre-release, diet post-release, environment pre-release, and environment post-release. All pairwise comparisons resulted in significant differences ($p<0.05$) between these groups for both metrics. (Figure 6; Supplementary Table S8–S9).

Intra-individual changes in headstart lizard gut microbiomes were visualized with a volatility plot, which displays the breadth of change (intra-individual Weighted or Unweighted UniFrac distance) in an individual headstart's gut microbiome on a monthly basis from January to July 2023 (Figure 7). We were most interested in how the transition from captivity to the wild (occurring from April to May) might induce change in the gut microbiome. It appeared that some headstart lizards experience increased differentiation throughout this transition, as demonstrated by line graphs with an upward slope between these points (Figure 4). However, it is clear that even in captivity, a very stable environment, an individual's gut microbiome may still be experiencing changes in community composition and structure. It is important to note that due to the poor sequence quality of some headstart lizard gut microbiome samples, most individuals do not have representative gut microbiome samples for every month included in the analysis.

Recommendations:

ASSESSMENT & PRIORITIES RELATED TO OBJECTIVES 1 & 2

This study examined the variable ways different age-classes of Texas Horned Lizards respond to headstarting and subsequent soft release into an existing urban population. Our results indicate that the headstarted adults were sufficiently thermoregulating and foraging because they all survived the soft release period, most survived approximately three months, and nearly all mortalities were caused by predation rather than desiccation, overheating, or starvation (Supplementary Table S1, Figure S1). Additionally, surviving adults underwent brumation, and two individuals performed natural reproductive behaviors the year following their wild release in 2024. However, compared to the resident population, the headstarted adults were characterized by smaller body sizes, reduced spatial movement patterns, and lower survivorship.

Survival of reintroduced animals depends largely on maintaining energy reserves necessary for homeostasis and growth (Kleiman 1989). In our study, headstarted adults never experienced extensive weight loss; however, individuals of neither sex reached the body sizes of the corresponding sexes in the resident group. While six lizards experienced moderate gains, five individuals remained within ± 0.5 g of their starting weight, and the individual excluded from spatial analysis, identified as ID_1083, lost nearly 2 g by the time it reached brumation. However, we note that during the 2023 active season we aimed to reduce handling stress by recording morphometrics primarily during tag changes, which prevented the collection of fine-scale growth trends.

Another desired outcome of a translocation project is for relocated animals to perform the natural spatial behaviors necessary to forage, naturally interact with conspecifics, and avoid predation in the wild (Kleiman 1989, Berger-TAL and Saltz 2014). Usually, researchers are concerned with increased mortality risk if the translocated animals released into unfamiliar territory make erratic movements or exhibit low site fidelity by dispersing far away from the release site (Reinert and Rupert 1999, Plummer and Mills 2000, Germano and Bishop 2009). This was not observed in our study, in fact, our findings align with previous research at TAFB in which translocated wild adults moved shorter distances per day than residents and did not make large homing movements (DeGregorio et al. 2020). Most of the headstarted adults exhibited reluctance to explore much of the available reserve habitat as evidenced by the clustering of their home ranges near the release site (Figure 1).

The headstarted group experienced a marked decline in survivorship due to mass predation in August, during which six lizards were depredated (five within just two tracking days) along the fence line delineating the northern boundary of the reserve, 50 m from the release pen (Supplementary Figure S1). The fence perimeter did not function as a physical barrier to animal movement, as the horizontal beams connecting the posts were 90 cm above the ground. It did, however, separate the tall prairie vegetation on the reserve side from the mowed neighborhood turf grass on the other side, creating a habitat edge. On the rare occasions when a lizard moved beyond this border, we would transfer them back to the reserve side to prevent human-induced mortality. Based on the small dentition marks found on the recovered transmitters, we believe striped skunks, possibly a single individual who repeatedly traveled along the fence, to likely be responsible. Additional transmitters from two headstarted individuals also were recovered along this stretch of fence; however, we could not reliably determine each individual's fate, as the tags did not show signs of damage and may have simply been shed.

The results from the release of headstarted adults are likely the culmination of husbandry conditions experienced before reintroduction and characteristics of the urban release site. While in captivity the headstarted adult lizards were confined for nearly 1.5 years in ~75 L enclosures that contained refugia in the form of cholla wood, artificial plants, and sandstone rocks. Although the physical features were used by the lizards, they were not particularly similar to refugia they would encounter at the release site, and the enclosure space limited their possible environmental interactions. As animals develop, they may modify their behaviors to better suit their current habitat, which in turn can drive future habitat selection preferences and locomotion performance (Aubret and Shine 2008). This behavioral plasticity might have conditioned the headstarted adults to utilize less of the available habitat post-release. Husbandry conditions, such as simplistic enclosures or longer duration in captivity, can also reduce captive animal competency in performing fitness-related activities (Almli and Burghardt 2006, Degregorio et al. 2013, Mack et. al. 2018). Abnormal spatial movement patterns or maladaptive behaviors can hinder proper habitat selection, inhibit resource acquisition, and increase predator exposure, potentially leading to poor body condition or death for translocated animals (Roe et al. 2010, DeGregorio et al. 2017). Additionally, although precautions were made regarding the headstarted animals' welfare, stress from long-term confinement in captivity and/or subsequent tracking post-release may still impact the health and subsequent growth of released individuals (Teixeira et al. 2007, Breed et al. 2019).

In our study, we could not confidently identify any clear association between the spatial movement metrics measured and the headstarted adults' body condition. Interestingly, in 2024, ID_1083 regained the weight lost before brumation and made further gains totaling ~5 g. It also attempted to mate and demonstrated spatial movement patterns similar to those of the resident adults. Similarly, before being depredated in 2023, three of the five individuals whose final weights were similar to their initial weight were in the process of regaining mass after post-release losses. Since most of the headstarted lizards did not experience dramatic weight loss and resident adults were using the habitat near the release pen, the environment evidently provided necessary thermal refugia and sufficient prey. Thus, we tentatively presume that despite some atypical movement patterns and lower average body sizes among headstarted adult lizards, they remained competent foragers. However, their lower daily movement rates likely limited encounters with residents during the mating season (May – June), as the latter were typically located farther into the reserve. The headstarted adults' window to mate with residents was already shortened, as we could not transport them to TAFB until May 5th due to poor weather conditions and they required several weeks in the soft release pen to acclimate. Unfortunately, this nullified one of the main benefits of releasing sexually mature animals: the potential to reproduce in their first year of reintroduction.

The primary pitfall associated with our release of headstarted adult Texas Horned Lizards, as with many headstart programs, was lower post-release survivorship resulting from a high number of predation events. Despite their morphological adaptations for crypsis, wild *P. cornutum* do still face significant predation pressure, with survivorship estimates varying across its geographic range: 0.59 – 0.70 & 0.57 (CI = 0.32 – 0.93) at TAFB (Endriss et al. 2007, DeGregorio et al. 2020); 0.09 – 0.54 in southern Texas (Fair and Henke 1999); and 0.35 – 0.86 in southern Arizona (Munger 1986). Our survival rate estimate for the headstarted adult cohort in 2023 was lower than that of the resident lizards at TAFB, but within the ranges reported in other studies. Unfortunately, by the end of the 2024 field season, there were no known survivors: one headstarted adult had to be euthanized post-brumation due to a severe cloacal infection, two others were found decapitated, and one was censored due to signal loss.

Surprisingly, despite having access to the entire reserve, during August 2023 nearly one-half of the headstarted adult lizards were residing alongside the northern fence boundary of the reserve, of which six were depredated. This dispersal towards the perimeter may have been stochastically driven due to the location of the release pen, or through selection for habitat structures contained within the habitat edge by the lizards. Regardless of the reason, such man-made linear features can increase the mortality risk for a prey species by increasing the perimeter-to-area ratio of habitat patches or serving as corridors that facilitate animal movement, thereby amplifying their exposure to predators (Barrows et al. 2006, Wolf et al. 2013, Hansen et al. 2019, Dickie et al. 2020).

In the wild, horned lizards rely not only on crypsis to avoid predators but also will initiate flight behaviors to escape imminent threats to their survival (Cooper and Sherbrooke 2010*a*, *b*). Prey naïveté, or the inability to recognize predators, can reduce survivorship by altering a prey species' antipredator behaviors, such as shortening their flight initiation distances (FIDs). This has been observed in other species in populations isolated on islands, individuals living in predator-removed environments, and domesticated populations (Brokordt et al. 2006, Cooper et al. 2014, Ross et al. 2022). Consequently, there are concerns that headstarting animals in complete isolation from predators during critical periods of their ontogenetic development may negatively impact their post-release survivorship (Tetzlaff et al. 2019). Most headstarted adult lizards in our study survived for several months post-release and were likely exposed to predators during that time; however, they did not receive predator recognition training while in captivity.

Perhaps more important for urban lizard populations is their perception of humans as predators. While many species can habituate to human disturbance, those that cannot may suffer negative impacts to fitness-related activities due to heightened fear responses (McGowan et al. 2014, Pellitteri-Rosa et al. 2017, Putman et al. 2024). For these species, fear can manifest in earlier escape attempts (greater FIDs), staying closer to refuges, or increased hiding, which can lead to poor body condition (Garrido and Pérez-Mellado 2015, Putman et al. 2024). Anecdotally, we observed that some headstarted adults appeared to respond to researcher approach by fleeing early rather than relying on crypsis. Premature fleeing can increase the likelihood of a predator visually detecting prey, and while this poses a cost to any prey species, it stands to reason it would be worse for a species whose morphological adaptations are for camouflage, not high-speed escapes (Cooper and Sherbrooke 2010*b*). Because crypsis would not have prevented human capture during captivity (or when necessary post-release), it is possible some individuals shifted towards initiating escape behavior earlier when threatened, though we did not test this hypothesis. We are uncertain whether the high mortality rate of the adult headstarts was due to phenotypic shifts in their anti-predator behavior in either direction (e.g. shorter or greater FIDs), the reserve edge acting as an ecological trap, unfortunate circumstance, or some other unknown factor. Regardless, personnel involved in headstarting programs must consider core reintroduction objectives and minimize unnecessary physical interactions that could increase stress in the captive animals or disrupt natural behaviors during subsequent post-release field monitoring (Teixeira et al. 2007, Berger-Tal et al. 2020).

In contrast to the headstarted adult cohort, the headstarted juveniles demonstrated positive responses to the post-release metrics used in this study, with some caveats owing to our small sample size. Our results indicate that the headstarted juveniles' spatial movement patterns were comparable to those of the residents, despite being nearly twice as large as their wild counterparts at the start of the 2024 season. The most parsimonious model for the log-transformed daily movement rates was the null model, revealing that not only was there no effect of *origin*, but that the *days* variable also was an uninformative predictor. Therefore, even though the resident juveniles were growing larger as the season progressed, movement rates were not dependent on body size; thus, we conclude that the headstarted juveniles demonstrated natural movement patterns typical of this urban population (Table 2). Similarly, we did not detect a significant difference in home range sizes between the two groups, regardless of the estimator used in our analysis.

Interestingly, the juvenile cohort moved away from the release site to occupy the core range utilized by residents more readily than the adult cohort. During the 2024 season, researchers decided not to immediately relocate individuals attempting large dispersal movements away from the reserve, opting instead to continue tracking to see if they could successfully emigrate to other potential habitat fragments at TAFB. Three juveniles attempted these dispersals, including one headstart and one resident, both of which occurred in early August after spending weeks in their small, separate home ranges. We rescued the headstarted disperser from a storm drain and relocated the other individual back to its home range, believing a human had transported him. This likely resulted from voluntary movement, as the next day the lizard again dispersed outside the reserve, albeit in a slightly different direction. Unfortunately, we lost the signal from this individual and one of the two resident dispersers, though this suggests that ontogenetically influenced dispersal behavior in Texas Horned Lizards is retained even if an individual is kept temporarily in captivity. Typically, overdispersion is considered a negative outcome in translocation projects (Berger-TAL and Saltz 2014). In this case, however, the lizards from both groups were well-established in their home ranges before making these exploratory excursions. Although there is inherent risk for animals traveling long distances, especially through urban matrices (e.g. falling in storm drains), some subadult dispersals may benefit fragmented populations through colonization of new habitat patches or gene flow with geographically proximate subpopulations.

A striking difference between the headstarted and wild juveniles was the discrepancy in their masses at the time of soft release. Hatchlings at TAFB reach an average weight of 0.9 ± 0.4 g before entering brumation, which increases to 1.8 ± 0.5 g the month after they emerge from their burrows as juveniles (Vesy et al. 2021). Despite having constant environmental conditions and feeding opportunities throughout the overwintering period in captivity, the headstarted individuals decreased their activity from December through February and experienced only modest weight gains (2.87 ± 0.64 g to 3.36 ± 0.81 g). However, from late March to May, their weight nearly doubled (5.45 ± 1.29 g to 10.31 ± 1.82 g), making them far larger than the two approximately 2.5 g residents we were tracking at that time. The headstarted juveniles' larger body sizes, and associated increases in horn and spine lengths, ultimately did not protect them from larger predators, as four were depredated within the 35 days post-release. Still, the increased mass during subadult ages may provide advantages by enabling predation on larger, more nutritious prey species, promoting faster growth rates at later life stages, and enhancing resistance to translocation-related stressors and stochastic environmental conditions deadly to smaller individuals (Madsen and Shine 2000, Alberts 2007).

Conservative estimates of Texas Horned Lizard hatchling survivorship from previous research at TAFB ranged from 0.25 – 0.51, though true survival rates were likely higher, as individuals with unknown fates were assumed deceased for this estimate (Vesy et al. 2021). Eight out of the nine hatchlings collected for this phase of the study survived captivity, confirming that headstarting effectively reduced their mortality risk during this age class. By the conclusion of this study, we did not detect a difference in survivorship between headstarted and resident juveniles, though both groups had lower seasonal survival than the wild adults. While these results are consistent with previous demographic and translocation research at our study site, they should be interpreted with some caution (Wolf et al. 2013, DeGregorio et al. 2020). Our sample size for the juvenile age class was limited, and we only tracked a single headstarted lizard to brumation, compared to four resident juveniles. The leading cause of mortality for headstarted juveniles was the unusual decapitations observed throughout the 2024 field season. However, we found no evidence to suggest maladaptive antipredator behaviors to have contributed to this outcome, as the decapitations were distributed across life stages and origin groups.

As urbanization continues to fragment and degrade wildlife habitats, conservation efforts will increasingly need to focus on habitat patches within urban matrices that can support declining populations (Brown et al. 2024). A meta-analysis of squamate responses to human-mediated habitat modification suggests that members of the family Phrynosomatidae may be better equipped to persist in these disturbed areas compared to other species (Doherty et al. 2020). Indeed, our study population of Texas Horned Lizards was able to persist on the reserve despite a reduction in their available habitat caused by the construction of residential housing (Wolf et al. 2013). Numerous small towns in Texas also support resident populations; however, compared to those living in natural areas, these urban populations are more isolated, have reduced genetic diversity, and have lower effective population sizes (Wall et al. 2024). The lack of gene flow between populations or sub-populations is largely due to the lizards' limited dispersal ability, which is further restricted by incompatible anthropogenic surroundings (Sherbrooke 2003, Wall et al. 2024). Lower genetic diversity can lead to a loss of adaptability, with important ramifications for the long-term survival of these populations in future environments (Williams et al. 2019). Additionally, these urban populations are highly dependent on thermal refugia provided by heterogeneous vegetation, and if removed, can experience population declines with lower likelihoods of recolonization (Tucker et al. 2023).

Interestingly, population estimates of Texas Horned Lizards residing in small urban areas have revealed they can occur at much greater densities than in non-urban habitats, possibly due to reduced predation pressure (Ackel 2015). To better understand this hypothesis, research comparing predation attempts on foam Texas Horned Lizard models placed in two Texas towns and a nearby rural ranch found significantly more attacks on the ranch, namely by avian and unknown predators (Mirkin et al. 2021). However, the immobile models were not well-suited for capturing attempts made by snakes, and cats were recorded ignoring the models, despite being known predators of reptiles in urban areas (Loss and Marra 2017).

Previous research on our study population also suggested that, given the urban surroundings and TAFB's regulations against free-roaming pets, predation pressure was likely lower than in rural populations (Vesy et al. 2021). Unfortunately, this was not the case during our study. In the fall of 2024, the TAFB Natural Resource Office responded to the numerous lizard decapitations by setting small mammal traps along the northern perimeter of Reserve 3 to remove any cats dwelling nearby. The traps captured multiple native mesocarnivore species known to inhabit the base, including opossums, raccoons, and skunks. Cats also were successfully trapped, confirming that either the pet policy was being ignored by residents of the adjacent neighborhood or stray cats were entering from outside the base.

If cats were indeed responsible for decapitating lizards, they would have accounted for approximately one-third of the total deaths documented in this study. Introduced cats are known to have detrimental impacts on reptile populations globally, so we find it unlikely that the Texas Horned Lizard's antipredator behaviors and morphological adaptations provide the same level of protection against cats compared to native predators (Loss and Marra 2017). In addition to the mortalities, five resident adults were censored in May and June 2024 due to signal loss, which represented an unusually high number of transmitter failures. This period coincides with the lizards' breeding season, so it is possible they moved outside the range of our receiver in search of mates, despite our extensive searches of Reserve 3 and the adjacent neighborhood. However, it is also possible that signal loss occurred via predators removing lizards from the reserve. Given the lack of immigration into this isolated population, its stability will largely depend on maintaining sufficient annual survival rates.

The field of conservation increasingly relies on the translocation of wild-sourced or captive-bred individuals to slow declines in threatened populations or reestablish lost populations. Translocation can be a versatile tool to meet these conservation objectives across taxa. Unfortunately, it is often applied without the necessary post-release monitoring (Escobar et al. 2010), which, when it has been performed, has revealed mixed programmatic success (Germano and Bishop 2009, Kraus et. al. 2017, Pérez-Buitrago et. al. 2008). Even if short-term translocation aims are met (e.g. satisfactory survivorship, performance of natural behaviors, or reproductive success), the realization of long-term population-level goals is not guaranteed (Germano and Bishop 2009). In this study, we identified factors likely to contribute to desired Texas Horned Lizard reintroduction outcomes and highlighted several key considerations essential for future headstarting initiatives. Our soft release methods proved suitable for both cohorts of headstarted lizards. However, it is likely the post-release behavior of headstarted adults was influenced negatively by the length of time they spent in captivity. It is possible that under different husbandry conditions these abnormal behaviors may not have been present or as pronounced. We suspect that natural spatial movement behaviors are more likely to be retained if headstarted lizards are housed in more spacious outdoor enclosures during early development to more closely emulate their release site environment and climate. Physical structures that closely mimic natural substrate and vegetation, along with the stimulus from nearby conspecifics, may assist in the recognition of refugia and microclimates, and encourage appropriate intraspecific interactions post-release (Stamps and Swaisgood 2007).

Although there is little research on predator avoidance training for reptiles, young, semi-captive El Hierro Giant Lizards (*Gallotia simonyi*) demonstrated shifts in basking and locomotion behaviors after undergoing repeated training trials using kestrel and cat models (Burunat-Pérez et al. 2018). We are not confident that abnormal antipredator behaviors resulted in decreased survivorship among headstarted lizards in our study, but the applicability of this method to increase post-release survival might be explored for future horned lizard reintroductions (Tetzlaff et al. 2019). Our study also used only a single release site near the portion of Reserve 3 most heavily used by the residents during the time of our study. Although *P. cornutum* have been found throughout the reserve over the previous 25 years, this reintroduction site decision was made to prevent anthropogenic disturbance during release, to account for our small sample size, and to facilitate the successful integration of headstarted lizards into the population. Had the headstarted adult cohort been released elsewhere, or released at multiples sites throughout the reserve, they may have been less susceptible to localized mass predation events. Finally, if weather conditions are agreeable, we recommend opening soft-release enclosures for headstarted adults fully before the beginning of their breeding season to increase chances of finding a mate the same year of release.

Based on our findings, juvenile Texas Horned Lizards may be more suitable candidates for future headstarting initiatives. The extent of the headstarted juvenile cohort's pre-release growth was unexpected, but most of that growth was concentrated in the two months prior to their release in May 2024. This accelerated growth was most likely a consequence of consistent foraging opportunities provided while in captivity, but the size of feeder insects also may have been influential. Once the lizards reached larger sizes, mealworms and small crickets were provided, which are generally larger than the large-bodied native ant species fed upon by wild Texas Horned Lizards at TAFB (Ramakrishnan et al. 2018). Although our headstarted juveniles showed no difficulty acquiring native prey species or growing post-release, care must be made to prevent impaction while horned lizards are kept in captivity.

Research on Smooth Greensnakes (*Opheodrys vernalis*) found that headstarted snakes that underwent brumation had rapid compensatory growth during the first two months of their active season, resulting in similar mean body sizes as snakes that were kept active year-round (Sacerdote-Velat et al. 2014). Consequently, recommendations were made to include brumation cycles during reptile headstarting efforts as a precautionary measure to preserve natural behaviors. The lizards at TAFB typically enter brumation in October and emerge between late March to April, though timing varies across years and individuals. Therefore, horned lizard headstart programs might encourage natural brumation behaviors while still achieving desired pre-release body sizes by slightly truncating their overwintering period (i.e. November to March). Furthermore, headstarted individuals will have less opportunity to form maladaptive behaviors if they are kept inactive for several months, especially if human handling is limited. Construction of enclosures containing natural habitat cues also should require less effort and financial investment given the hatchlings' small stature and limited home range.

Future research should focus on acquiring larger sample sizes and comparing releases of headstarted hatchlings to those that are raised to juvenile age, to determine if accelerated growth and reduced predation risk in early spring translates to a greater number of lizards surviving to sexual maturity. Given the Texas Horned Lizard's limited dispersal abilities, careful consideration must be given before selecting insular urban environments for population augmentation. Predation risk should either be evaluated through surveys of the local fauna and identification of edge habitat or reduced through predator-removal initiatives if appropriate (Lettink and Armstrong 2003, Phillips et al. 2005, Hansen et al. 2019).

Management Implications:

The augmentation of at-risk populations through headstart programs depends on thorough post-release monitoring to identify factors that enable the realization of short-term objectives. If headstarted animals are to reinforce existing populations or establish ones, they must first competently perform fitness-related behaviors similar to their wild counterparts. Findings from our study support the use of soft release techniques during Texas Horned Lizard reintroduction but suggest that refinement of headstarting husbandry protocols is warranted. Areas that could be improved include time spent in captivity, enclosure conditions and complexity, handling frequency and predator recognition training, and replication of natural life cycles. Current headstarting initiatives for Texas Horned Lizards release captive-bred hatchlings before brumation. Given that our headstarted juveniles were protected from predation until late May, experienced accelerated growth in captivity, and demonstrated natural behaviors once released, managers also should consider including juveniles into existing release protocols. Despite the growing need for urban conservation, we caution against urban release sites unless reintroduction is coupled with predator management strategies.

ASSESSMENT & PRIORITIES RELATED TO OBJECTIVE 3

Our study aimed to better understand the impact of conservation translocation on the Texas Horned Lizard's gut microbiome. First, to better understand how diet and environment shape the host-associated microbiome, we examined extrinsic microbial communities observed across captivity and the wild. Next, we comparatively analyzed the post-release headstart gut microbiome to wild residents to investigate if periods of captivity had a lasting impact on the host-associated microbiome. Finally, we assessed longitudinal changes in the headstart lizard gut microbiome associated with their transition from captivity to the wild to understand how the gut microbiome is affected by translocation practices. Overall, our study provides insight into the health and fitness of headstarted individuals during the post-release period. Conservation programs such as captive breeding and headstarting rarely incorporate post-release monitoring in any form, leading to an insufficient knowledge surrounding the survivorship, fitness, and microbiome changes of headstarted/captive bred individuals after their release (Germano & Bishop, 2009; Brown et al., 2021). Monitoring the gut microbiome of released individuals could be one way to obtain information related to the health and fitness of released individuals, especially when compared to wild resident individuals.

Contributions of Diet and Environment to Lizard Gut Microbiome in Captivity and the Wild.

It is well-established that changes in the diet and environment of an animal impact the structure and community diversity of the host's gut microbiome (Yuan et al., 2015; Jiang et al., 2017; West et al., 2019). However, it is much less clear if diet and/or environmental microbiomes possess a source/sink relationship with the gut microbiome (i.e., that the microbiota in the gut microbiome originate from prey items or local environments, and these microbes are then horizontally transmitted to the gut microbiome). Although it is plausible that a host consuming prey items or interacting with their environment provides a pathway for microbes to colonize the gut, the core microbiome is likely determined by the metabolic needs of the host, which change in response to dietary and environmental changes (Holmes et al., 2019). Other work on the squamate reptile gut microbiome provides mixed evidence for this source/sink relationship. A study on the gut microbiome of the omnivorous lizards *Liolaemus parvus* and *Liolaemus ruibali* found little to no overlap with the invertebrate microbiome or soil microbiome, whereas these microbiomes showed almost 40% overlap with microbial communities on plant material in the same environment (Kohl et al., 2017). Interestingly, in our study, we identified clear shifts in the taxonomic composition of the headstart gut microbiome post-release that were not mirrored by shifts in the microbiomes associated with the environment and diet. Post-release, the headstart gut microbiome was uniformly enriched with Actinobacteriota (increase in average relative abundance from 15.06% in captivity to 32.63% in the wild) while the proportion of Proteobacteria (54.28% in captivity to 29.88% in the wild) and Firmicutes (18.89% in captivity to 8.42% in the wild) decreased (Figure 4; Supplementary Table S2). When comparing the captive and wild environment microbiome samples, we observed a decrease in the average relative abundance of Firmicutes (18.66% in captivity to 2.18% in the wild), but the average relative abundances of Proteobacteria (30.69% in captivity to 32.27% in the wild) and Actinobacteriota (13.10% in captivity and 16.80% in the wild) were relatively consistent across these environments (Figure 4; Supplementary Table S2). Meanwhile, in the microbiomes of associated prey items, Actinobacteriota remained relatively consistent (2.48% in captivity and 3.90% in the wild), and we saw a decrease in the relative abundance of Firmicutes (41.13% in captivity to 4.93% in the wild) but a sharp increase in that of Proteobacteria (35.55% in captivity to 68.49% in the wild; Figure 4; Supplementary Table S2). These results suggest that the taxonomic composition of the headstart gut microbiome may not be directly associated with extrinsic microbial communities. In other words, moving from a captive to wild setting alters the host-associated gut microbiome in a manner that is not clearly reflected by corresponding shifts in the extrinsic dietary or environmental microbial communities.

Given that captivity presents a more stable and homogenous environment than the wild, it is plausible to assume that extrinsic microbial communities in captivity would contain lower levels of alpha diversity (within-sample diversity) than in the wild. We found that wild environmental microbiomes had higher Shannon diversity than captive environmental microbiomes, but we did not find a significant difference in operational taxonomic unit (OTU) richness (Supplementary Table S5). We note that the substrate used in the captive enclosures at the OKC Zoo was a mixture of sand, mulch, decomposed granite, and locally sourced soil. Since the Oklahoma City Zoo and TAFB are only about 10 miles apart, the locally sourced soil may be contributing to similar alpha diversity across captive and wild environmental microbiomes. We also were surprised to find no significant differences in the alpha diversity of captive and wild prey microbiomes (Supplementary Table S5). Captive prey items were primarily sourced from the online retailer Josh’s Frogs or the Oklahoma City Zoo’s stock, where they are exposed to minimal environmental variation and kept in stable conditions, leading us to hypothesize that the associated microbial communities would contain lower levels of diversity when compared to the wild prey items. However, previous studies of vertebrates have similarly identified no difference in alpha diversity across captive and wild populations (Scupham et al., 2008; Campos et al., 2018; Tong et al., 2019; Diaz and Reese, 2021). Additionally, a past study on the Comal Springs riffle beetle (*Heterelmis comalensis*) found that the microbiome of captive beetles was more diverse than wild beetles (Mays et al., 2021). These results are in contrast to the common notion that wild microbial communities are less homogenized than captive microbial communities (Dallas and Warne, 2022). However, it is important to consider that our wild diet and environment samples may have failed to capture the total diversity of extrinsic microbial communities present on Reserve 3. In the wild, we were only sampling a portion of the possible habitat and prey items utilized by the lizards, whereas in captivity we were able to more effectively sample the entirety of the cage environment and all prey items.

Texas Horned Lizard Gut Microbiome Composition:

Our investigation of the Texas Horned Lizard gut microbiome provides novel insight into the gut microbiome of squamate reptiles, an overall understudied taxa within the microbiome literature (Smith et al., 2021; Hoffbeck et al., 2023). Most host-associated microbiome work focuses on humans or mammals, while a limited number of studies feature reptiles as the focal taxa (Siddiqui et al., 2022). Further, even fewer studies have investigated the microbiomes of threatened reptilian species (Colston et al., 2017, Carranco et.al. 2022), leaving major gaps in our knowledge of the microbial community composition and structure of vulnerable reptile taxa.

Previous studies have suggested that Bacteroidota, Proteobacteria, and Firmicutes are core phyla of the reptile gut microbiome (Siddiqui et al., 2022). Across all lizard gut microbiome samples here, we identified these three core phyla, although at varying relative abundances. Proteobacteria had the highest relative abundance in pre-release headstarts when compared to post-release headstarts and residents (average relative abundance = 54.28%; 29.88%; and 31.45%, respectively; Supplementary Table S2). Similarly, the phylum Firmicutes was present at a greater abundance in pre-release headstart microbiomes compared to the other groups (18.89%; 8.42%; and 9.27% respectively). We found Bacteroidota to be present at lower relative abundances than compared to the other two proposed core reptile phyla, only present at relative abundances of 3.51% to 5.82% across lizard sample groups. Beyond these three phyla, we also observed Actinobacteriota at a high average relative abundance (>15%) across headstart and resident lizard gut microbiomes (Supplementary Table S2). Some other studies also have identified Actinobacteriota as a prominent phylum in lizard gut microbiomes (Tang et al., 2022; Vasconcelos et al., 2023).

The preliminary data for the gut microbiome of the first Texas Horned Lizard cohort released to the wild in 2021 appeared to be composed primarily of Bacteroidota and Firmicutes (both present at average relative abundances >25%) across pre-release, post-release, and wild individuals (S. Eliades personal communication). The lower average relative abundance of Bacteroidota within our lizard gut microbiome samples may be due to sample collection differences. We utilized cloacal swabs as a proxy for sampling the gut microbiome, whereas the previous study utilized fecal samples. A study on the gut microbiome of *Sceloporus virgatus* lizards found that fecal samples contained higher proportions of Firmicutes and Bacteroidetes, whereas cloacal swabs contained a higher proportion of Proteobacteria (Bunker et al., 2022).

Temporal Texas Horned Lizard Gut Microbiome Dynamics across Captive and Wild Environments:

Previous research on wildlife host-associated microbiomes has established that captive and wild populations differ in community composition and structure (Trevelline et al., 2019). However, much less is understood regarding how transitions between captive and wild settings may affect the host-associated microbiome over time. Most studies demonstrate that the gut microbiome of translocated individuals will restructure to be indistinguishable from wild counterparts over time (Chong et al., 2019; Schmidt et al., 2019; Yao et al., 2019; Korpita et al., 2023). However, the length of time for the gut microbiome to restructure varies widely; studies on the Tasmanian devil, deer mouse, and boreal toad suggest a restructuring period of two to four weeks, while research on the giant panda suggest up to a year was needed for the gut microbiome to lose signatures of captivity (Chong et al., 2019; Schmidt et al., 2019; Yao et al., 2019; Korpita et al., 2023). Differences in life history strategy and ecology among these species may contribute to variation in the gut microbiome restructuring period.

Within squamate reptiles, a study on the critically endangered Fijian crested iguana found that the gut microbiomes of translocated individuals took two months to be indistinguishable from the wild population (Eliades et al., 2021). Similarly, our results demonstrate that the gut microbiomes of captive headstart lizards restructure within two months post-release to more closely resemble the gut microbiomes of wild resident lizards (Figure 5). Less than one-month post-release, the gut microbiome of headstart individuals may still contain traces of the captive gut microbiome structure: headstart and resident lizards displayed significant differences in both alpha and beta diversity indices in May (Figures 2, 3). However, when headstart and resident lizards were sampled again in July (two months post-release), neither alpha nor beta diversity differed among these populations (Figures 2, 3).

It is interesting to note that the peak in alpha diversity of the headstart lizard gut microbiome was immediately following their translocation to Reserve 3 in May (Figure 5). One explanation could be that the gut microbiome of headstart lizards enters a hyper-diverse state distinct from both their own previous gut microbial communities displayed in captivity and the gut microbiome of their wild, resident counterparts. Linear mixed models demonstrated that the alpha diversity of the headstart lizard gut microbiome in May is significantly higher than all other months except June (Figure 5; Supplementary Tables S3, S4), and Kruskal-Wallis tests further supported this conclusion (Figure 5; Supplementary Table S5). Although studies on mammalian and amphibian taxa have not noted this immediate peak in alpha diversity post-release (Chong et al., 2019; Schmidt et al., 2019; Yao et al., 2019; Korpita et al., 2023), a study on the Fijian crested iguana also found the alpha diversity of post-release individuals to be higher than wild counterparts (Eliades et al., 2021). During this period, beyond adjusting to a new diet and habitat, the headstart lizards are experiencing other novel environmental factors like fluctuating temperatures and rainfall which could all contribute to variation in gut microbiome diversity (Moeller et al., 2020; Williams et al., 2022). The observed peak in alpha diversity may be due to the transitory state of the gut microbiome; microbes found predominantly in captivity may remain, while concurrently, microbes found in the wild are beginning to colonize the gut microbiome, leading to a high level of community diversity. Future studies may more closely examine gut microbiome assembly throughout this transitional period.

Management & Conservation Implications:

Given the relationship between the gut microbiome and animal health, it is important for conservation practitioners to consider how translocations between captive and wild settings impact animal fitness. Although our results demonstrate that the gut microbiome community composition and structure of translocated individuals does shift to match wild counterparts within two months post-release (Figures 2, 3), the in-between period of microbial community restructuring could have fitness consequences. Our beta diversity volatility plots demonstrate that the gut microbiomes of some headstart lizards display increased change in community composition and membership from April to May, when they transition from captivity to the wild (Figure 7). Additionally, this period coincides with a peak in headstart lizard alpha diversity, suggesting that new bacterial species are colonizing the microbiome during this time (Figure 5). Both findings suggest that the first month post-release could be a period of instability for the gut microbiome. A destabilized gut microbiome may derail beneficial physiological functions for the host, alter host behavior, or even hinder reproductive output (Desbonnet et al., 2014; Zaneveld et al., 2017; Comizzoli et al., 2021; Barathan et al., 2024; Sonnega and Sheriff, 2024). Future studies may more closely examine how gut microbiome variation and destabilization correlate with changes in host spatial movements or behavior post-release.

Conservation practitioners may consider implementing soft-releases practices to allow host-associated microbiomes of released individuals to restabilize and adjust to the new environment. Translocation soft-release practices involve constructing enclosures within the release area that allow individuals to adapt to the new habitat without the risk of predation (Resende et al., 2021). In this case, if individuals are at a fitness disadvantage due to the restructuring of the gut microbial community, a soft release would better support the survivorship of translocated individuals. In our study, the headstart lizards were placed in an outdoor enclosure for a three-week soft release period upon their release to TAFB in May 2023, which happen to coincide with the period of gut microbiome restructuring.

It is important to note that our study utilized 16S rRNA amplicon sequencing, which only provides insight into taxonomic community diversity. Without metagenomic sequencing of the gut microbial community, it is difficult to determine if the restructuring of the community composition and membership of the gut microbiome community also indicates a change in the beneficial physiological functions provided by the gut microbiome. Future studies should consider utilizing whole-genome or shotgun metagenomic techniques to better investigate the metabolic capabilities and functions of microbes within and across these communities. Although our study found evidence of a taxonomic restructuring period of the gut microbiome community, future work could investigate if this also corresponds with a functional restructuring of the gut microbiome.

Overall, our work provides insight into longitudinal microbial community diversity dynamics associated with host transitions from captive to wild environments. It is clear the animal gut microbiome is sensitive to environmental change and may take a variable period of time to restabilize after being introduced to a new habitat. Given the widespread anthropogenic environmental changes that animal populations are facing globally, and the physiological importance of the gut microbiome for host health, it is essential to better understand microbial community dynamics and its relationship to host fitness and survivorship.

Significant Deviations:

We did not complete the fourth narrative objective for this grant regarding the analysis of population-level genetic diversity and long-term genetic viability within the TAFB horned lizard population. We experienced several unforeseen setbacks during the first two years of the grant including delays caused by the global COVID pandemic and the unexpected departure of a trained and experienced graduate student during the summer of 2022. We were unable to train ourselves or recruit an experienced student, and as a result we stopped pursuing this objective in 2023 and redirected our resources toward objectives 1 – 3. We explored potential areas of collaboration with Dr. Dean Williams at Texas Christian University, who has an active, conservation-focused horned lizard research program, to help assess the genetic composition of the horned lizard population at TAFB, and we shared with his lab some of the tissue and blood samples that we had collected from the TAFB lizards during 2021 and 2022. He has informed us that his recent landscape genomic study, which is currently under peer-review, includes samples that we provided from the TAFB population and these have been integrated into his regional analyses.

Equipment:
No equipment exceeding $5,000 in cost was purchased for this project.

Overmatch:
University of Oklahoma (OU) has provided overmatch for this grant. OU did not request reimbursement for eligible grant expenditures which were in excess of the amount needed for non-federal match, resulting in overmatch.

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Table 1: Summary of mean daily movement rates over the study period for two age classes of headstarted and resident Texas horned lizards at Tinker Air Force Base, OK, USA. Movement rates are averaged from May 26th, 2023 – October 1st, 2023, for adult lizards and from May 20th, 2024 – October 1st, 2024, for juvenile lizards.

Individual daily movement rates [meters/day]					
	Origin	**N**	**Mean (SD)**	**Median (IQR)**	**Range**
Adults 2023	Headstart	15	7.48 (4.61)	6.19 (2.84)	2.66–19.94
	Resident	17	12.95 (4.70)	11.10 (6.95)	5.18–22.53
Juveniles 2024	Headstart	8	8.75 (3.91)	7.57 (3.24)	3.73–16.85
	Resident	9	10.80 (7.48)	9.38 (2.48)	4.01–29.57

Table 2: Summary of home range estimates over the study period for two age classes of headstarted and resident Texas Horned Lizards at Tinker Air Force Base, OK, USA derived from 95% minimum convex polygons and 95% kernel density using the plug-in bandwidth method. Estimates are from May 26th, 2023 – October 1st, 2023, for adult lizards and from May 20th, 2024 – October 1st, 2024, for juvenile lizards.

Home Range [hectares]								
			95 % MCP			**95% KDE**		
	Origin	**N**	**Mean (SD)**	**Median (IQR)**	**Range**	**Mean (SD)**	**Median (IQR)**	**Range**
Adults 2023	Headstart	13	0.13 (0.08)	0.13 (0.07)	0.04–0.32	0.14 (0.09)	0.11 (0.11)	0.01–0.30
	Resident	17	0.40 (0.41)	0.28 (0.24)	0.10–1.74	0.38 (0.31)	0.27 (0.08)	0.11–1.24
Juveniles 2024	Headstart	8	0.20 (0.20)	0.12 (0.35)	0.01–0.47	0.16 (0.16)	0.11 (0.10)	0.03–0.53
	Resident	8	0.53 (0.80)	0.13 (0.55)	0.01–2.02	0.21 (0.30)	0.12 (0.08)	0.03–0.94

Table 3: Summary of survival estimates using Kaplan-Meier estimation for two age classes of headstarted and resident Texas Horned Lizards at Tinker Air Force Base, OK, USA. The adult survival rates are calculated over the entire study period from April 2023 – October 1st, 2024. The juvenile rates are calculated from May 20th, 2024 – October 1st, 2024. The asterisk treats a headstarted juvenile's ambiguous mortality as deceased for the low survival estimate and right-censored for the high estimate.

Survivorship							
					Survival rates (95% CI)		
	Origin	**N**	**Mortalities**	**Censored**	**To brumation**	**Post-brumation**	**Second brumation**
Adults 2023	Headstart	16	11	5	0.35 (0.11–0 .60)	0.35 (0.11–0.60)	0
	Resident	18	9	6	0.65 (0.34–0.84)	0.65 (0.34–0.84)	0.35 (0.12–0.60)
Juveniles 2024	Headstart	8	6/5*	1/2*	0.19 (0.01–0.54) 0.38 (.09–0.67)*	NA	NA
	Resident	9	4	1	0.52 (0.16–0.80)	NA	NA

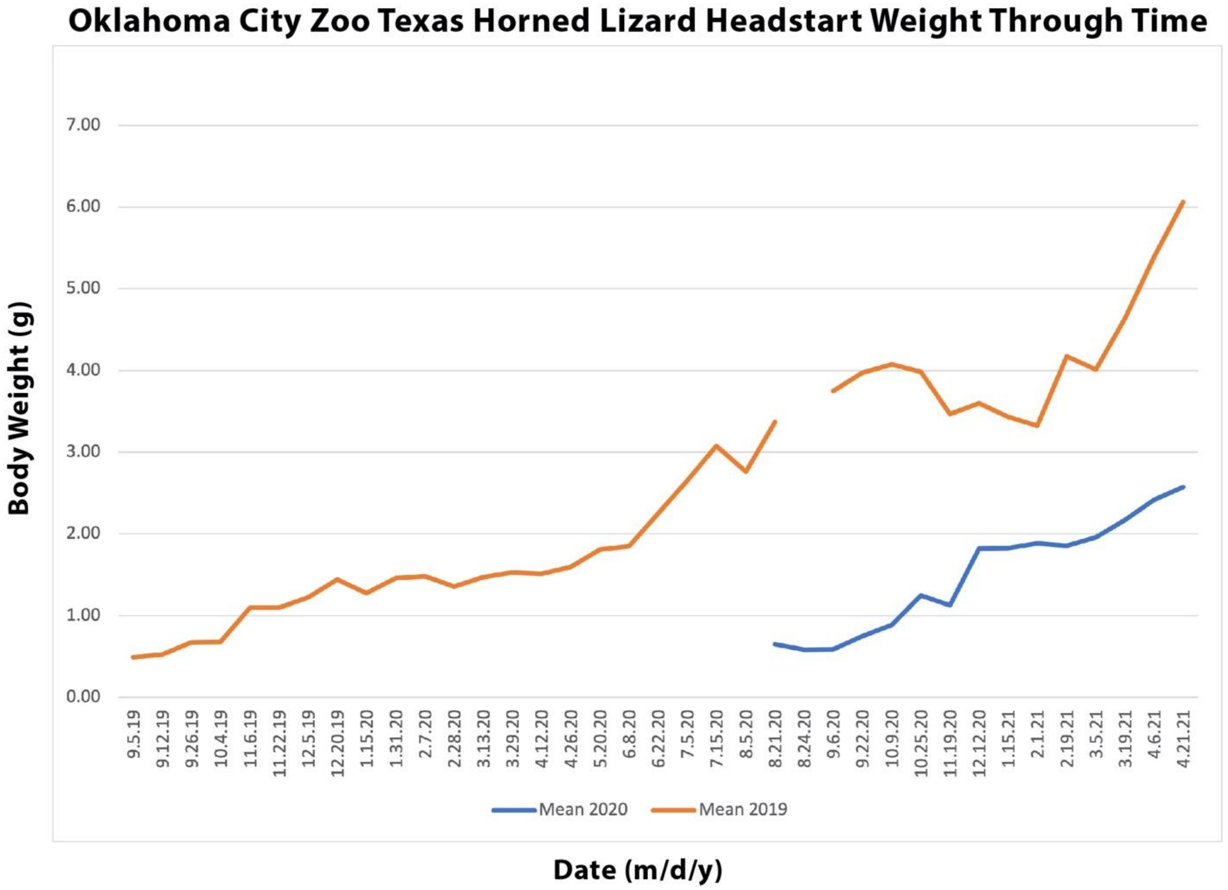

Figure 1. Example of annual monitoring of headstart Texas Horned Lizard mean body weight in grams (2020 and 2021) over time while in human care at OKC Zoo Lizard Lab.

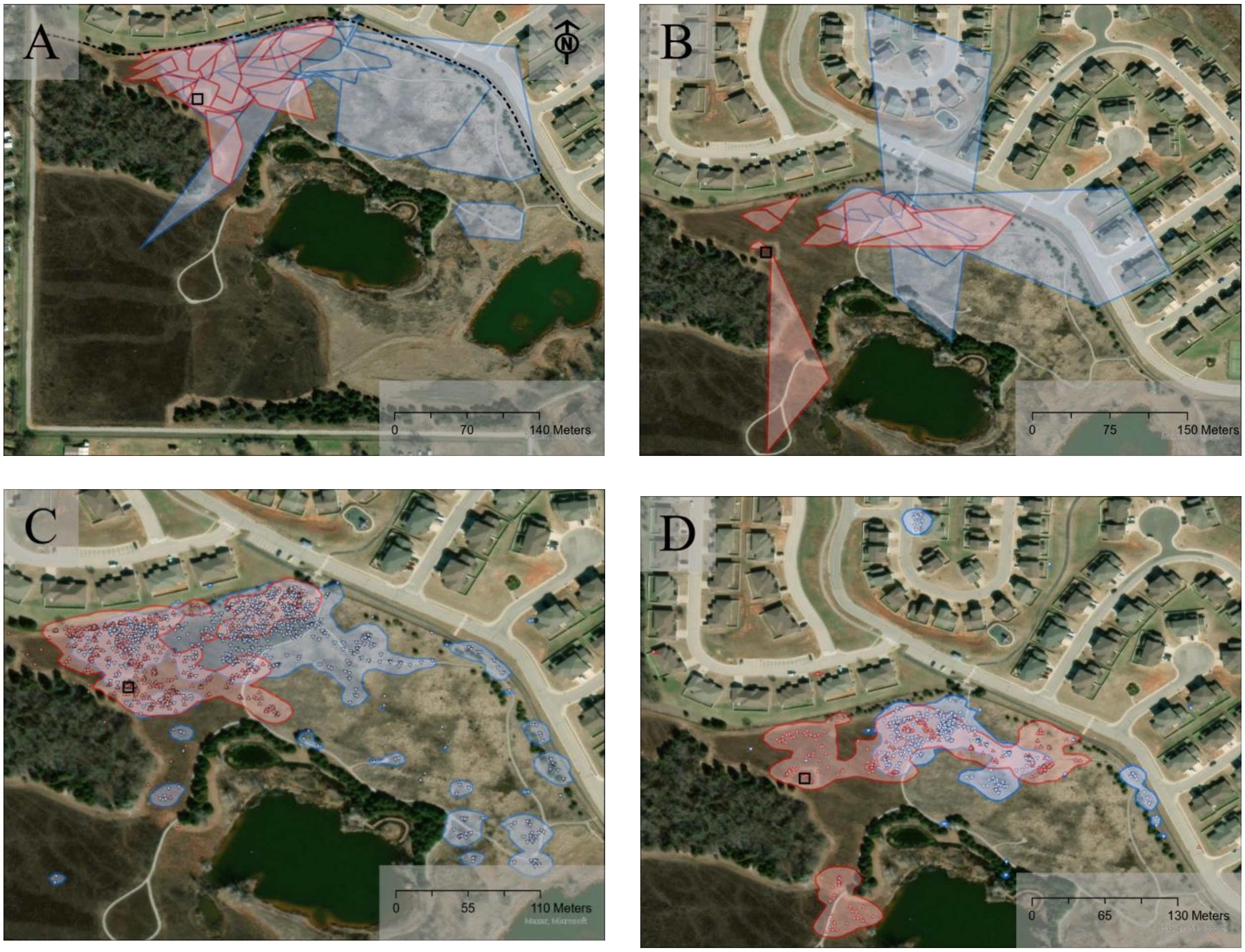

Figure 1: Area used by Texas Horned Lizards at Tinker Air Force Base, OK, USA. Individuals' home ranges outlined with 95% minimum convex polygons for **A)** adults in 2023 (headstarts: *n* = 13, residents: *n* = 13); **B)** juveniles in 2024 (headstarts: *n* = 8, residents: *n* = 8). Also, the total area used by headstarted, and resident lizards, delineated with 95% isopleths derived from kernel density estimation using the plug-in method, overlaid with pooled individual relocations (triangles) for **C)** adults in 2023 and **D)** juveniles in 2024. Red polygons and triangles represent headstarted lizards, blue represents residents. The black square represents the soft release pen, and the black dotted line in frame A represents the wooden fence that runs along the northern boundary of the reserve.

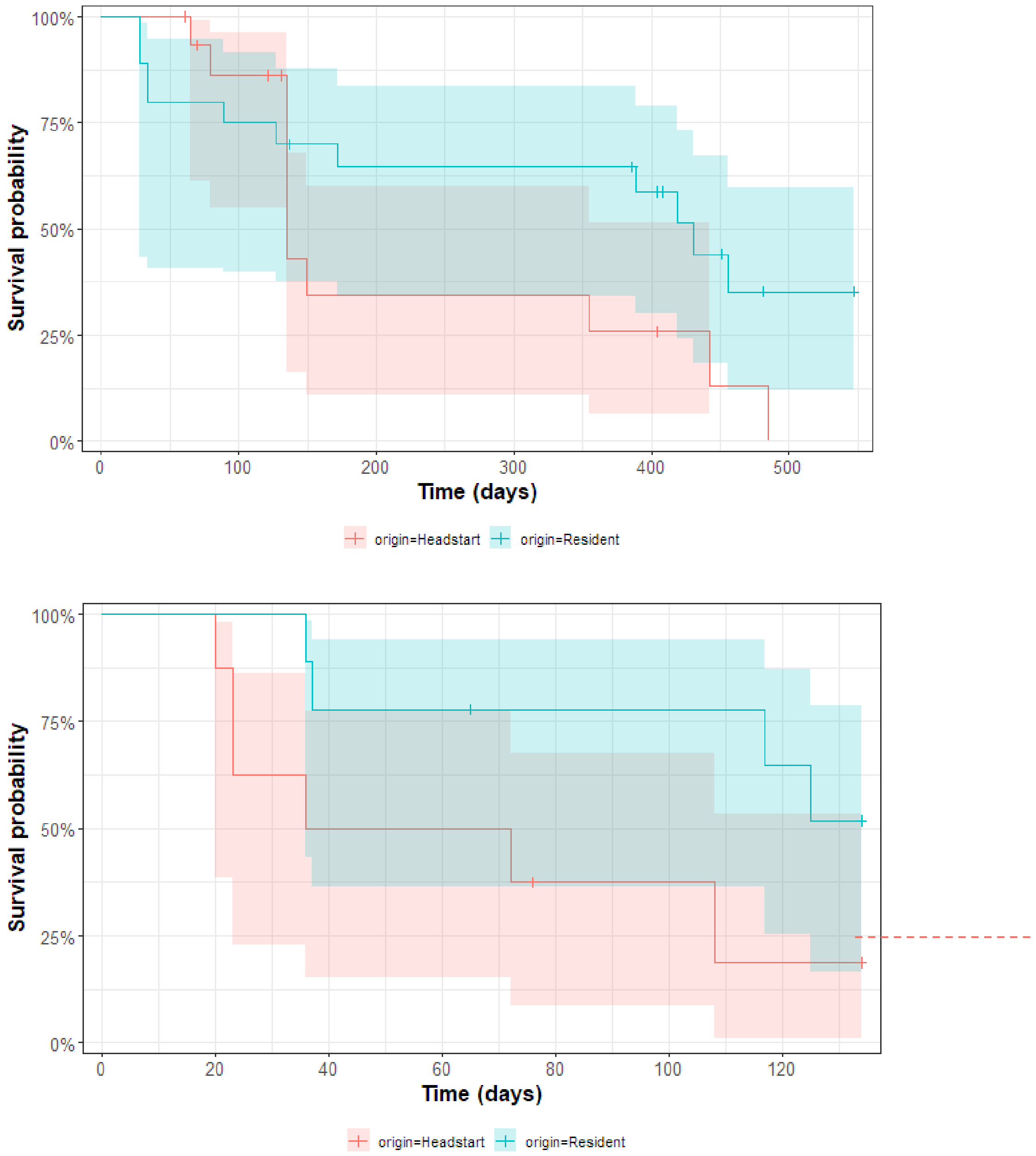

Figure 2: Kaplan-Meier survival curves for 2 age classes of headstarted and resident Texas Horned Lizards on Tinker Air Force Base, OK, USA; Blue shading represents 95% confidence intervals surrounding blue resident survival curves, red for headstarted survival curves for **A)** the adult cohort from April 2023 – October 1st, 2024 (headstarts: *n* = 16; residents: *n* = 19), and **B)** the juvenile cohort from (headstarts: *n* = 8; residents: *n* = 9). The dotted line represents the high estimate of survival for the headstarted juvenile cohort. Right-censored events are represented by tick marks.

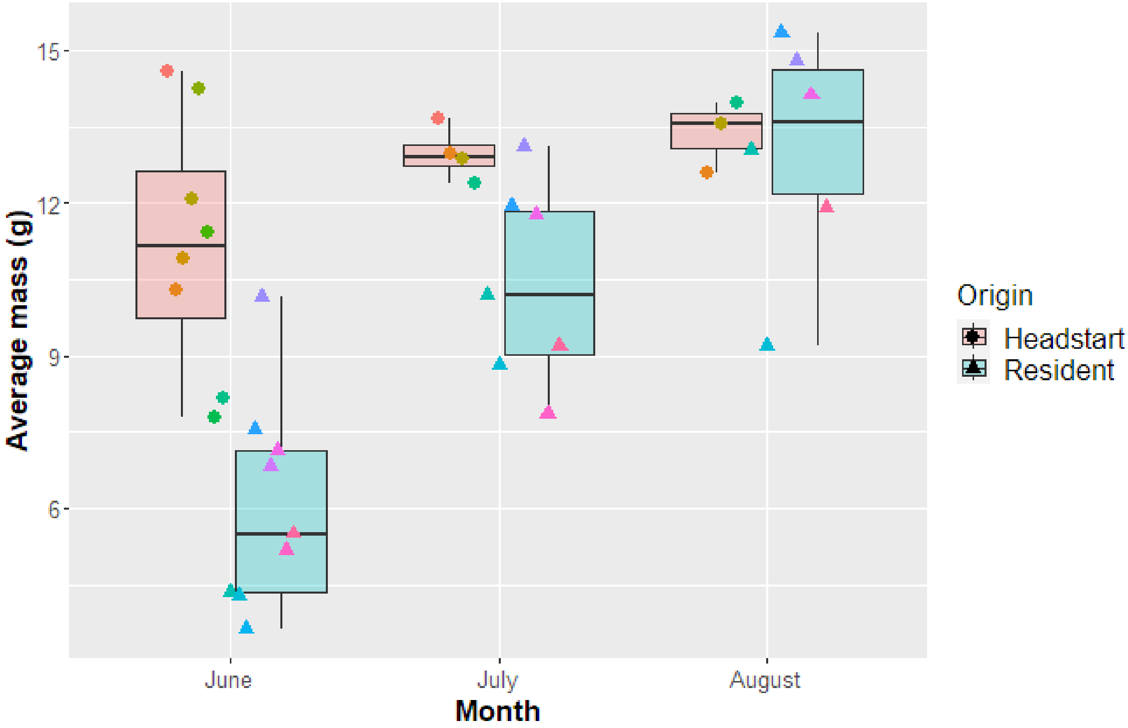

Figure 3: Average mass measurements for juvenile Texas Horned Lizards at Tinker Air Force Base, OK, USA during the 2024 study period. Colored circles or triangles represent individual lizards.

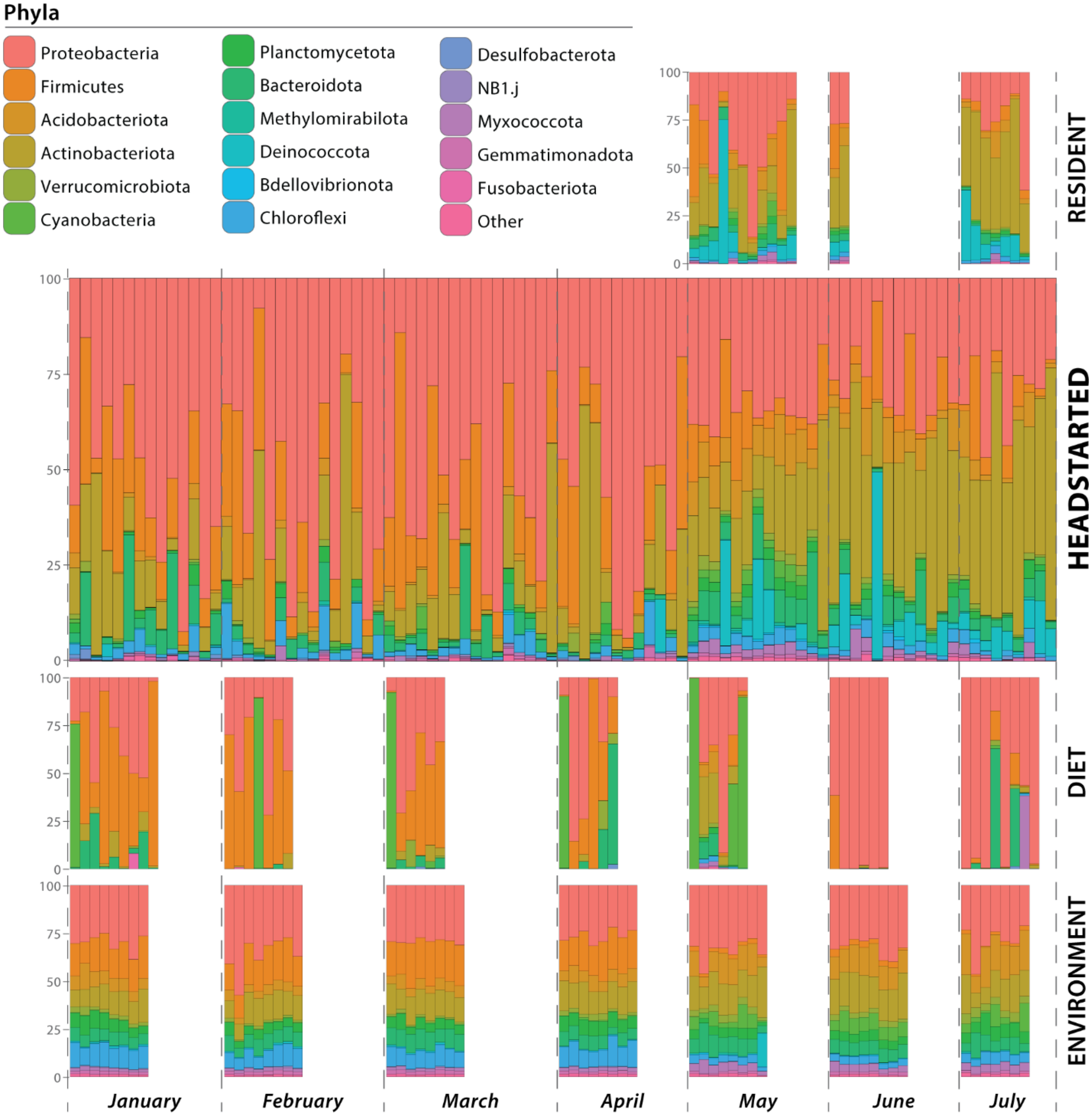

Figure 4. Microbial composition of samples at the phylum level. Samples are grouped by resident lizard gut microbiome, headstart lizard gut microbiome, diet microbial community, and environmental microbial community. Within each group, the samples are ordered temporally. Horizontal bars represent individual samples, and colors correspond to phyla.

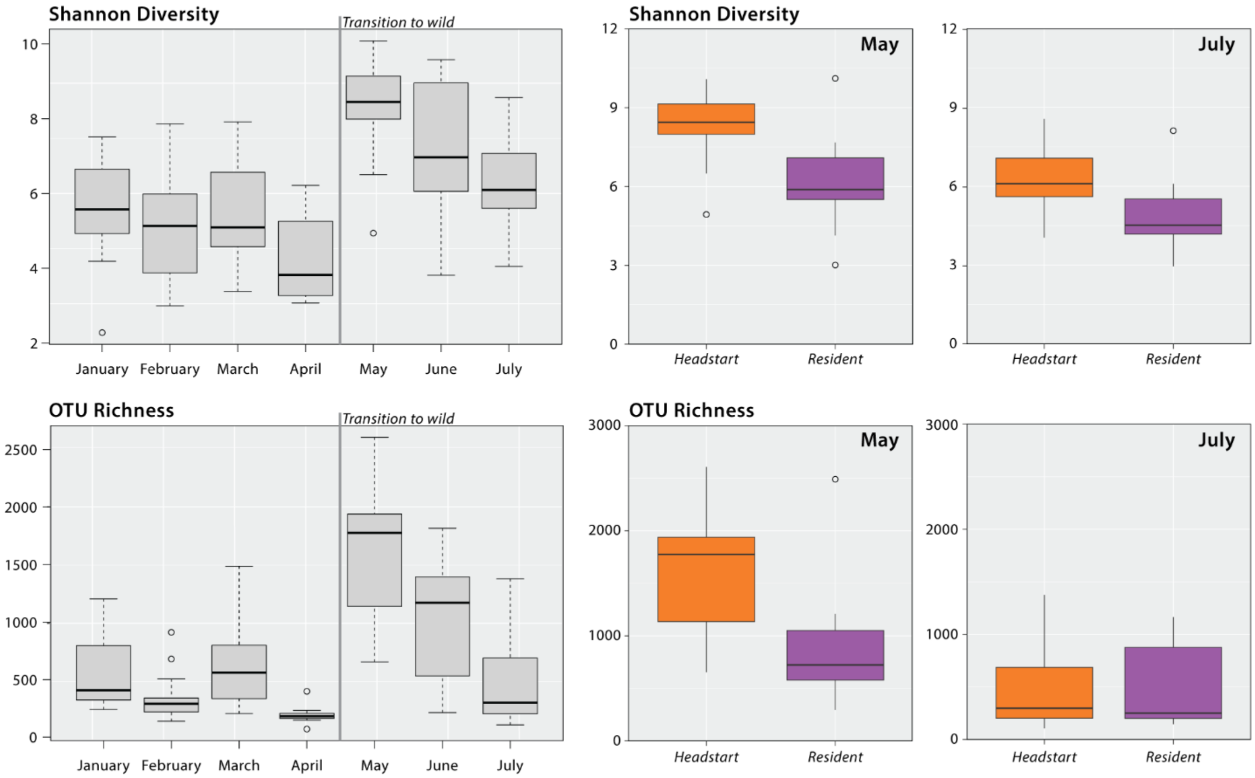

Figure 5. Alpha diversity analysis of headstart and resident lizards. Gray boxplots display the headstart lizard Shannon Diversity and OTU Richness by month from January to July 2023. Results of linear mixed modeling and post-hoc pairwise comparisons can be found in Supplementary Table S4 and S6. Colored boxplots display Shannon Diversity and OTU Richness comparisons of headstart and resident lizard gut microbiomes in May and July 2023. Results of Kruskal-Wallis tests can be found in Supplementary Table S7.

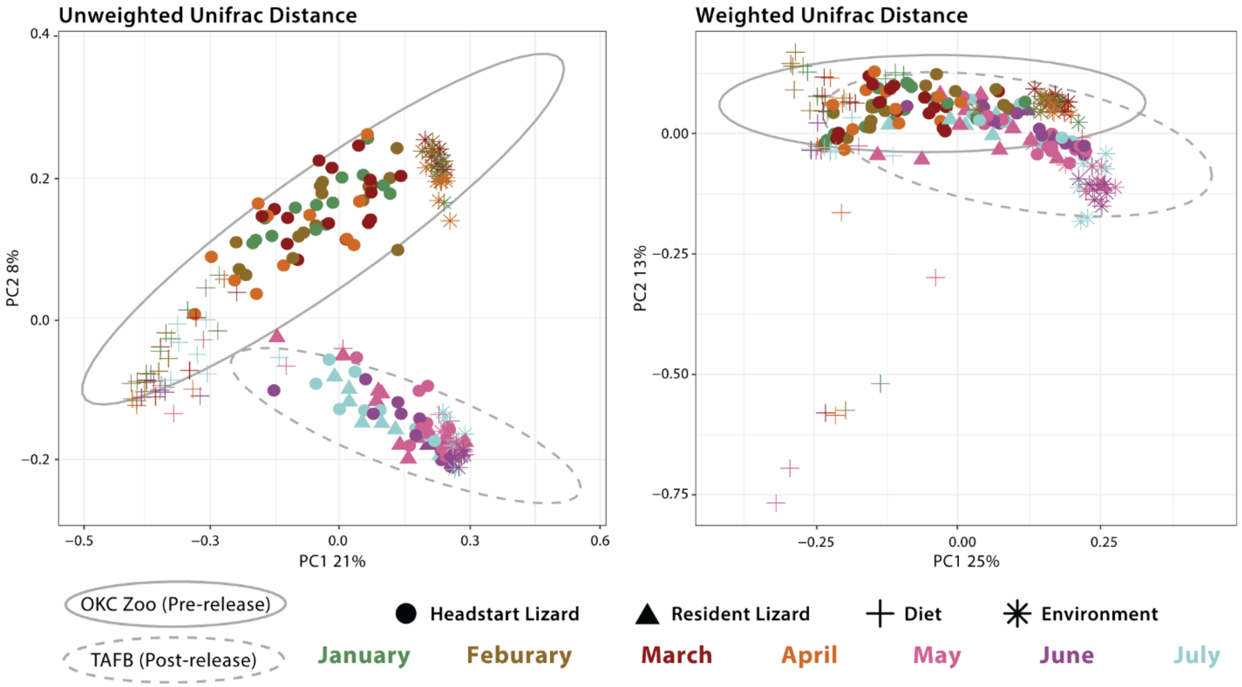

Figure 6. Principal Coordinates Analysis (PCoA) of all samples based on Unweighted UniFrac Distance and Weighted UniFrac Distance. Shapes represent different sample types: headstart lizard gut microbiome, resident lizard gut microbiome, dietary microbial community, and environmental microbial community. Colors correspond to the month of sample collection.

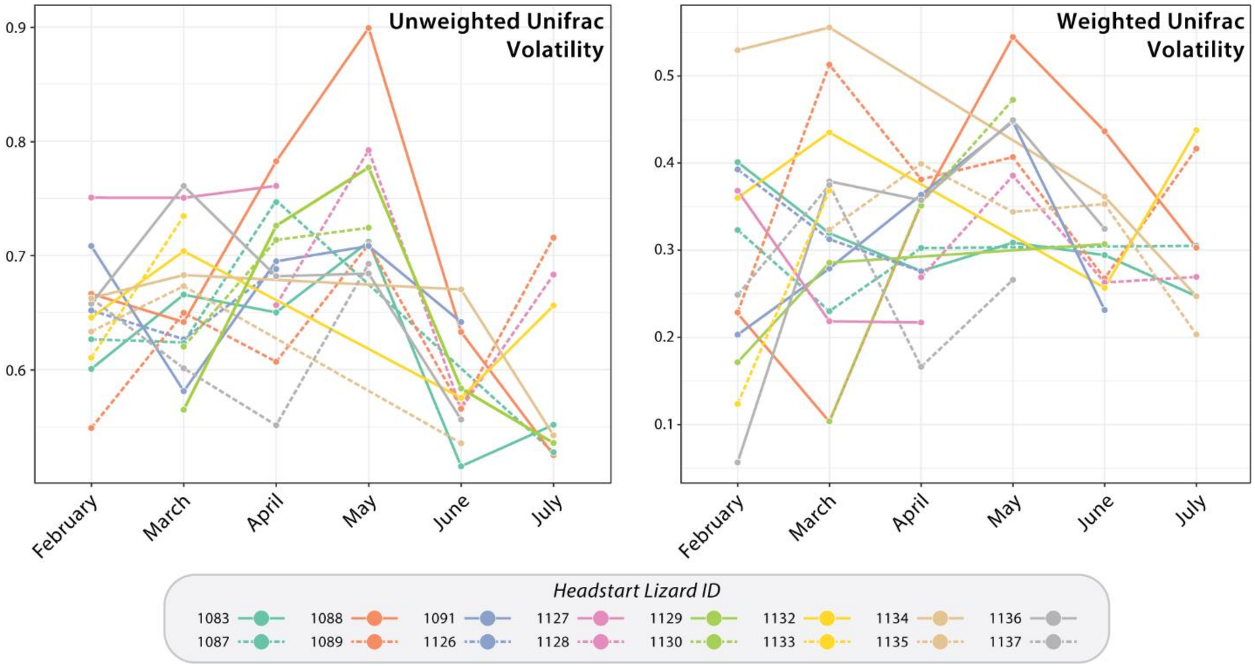

Figure 7. Volatility plots based upon Unweighted UniFrac Distance and Weighted UniFrac Distance of headstart lizard gut microbiome samples from January to July 2023. Each line graph corresponds to a headstart lizard and displays the breadth of change in their successive gut microbiome samples. An upward slope indicates increased differentiation of the gut microbiome between time points, whereas a downward slope indicates lower differentiation or less change in microbial community composition and structure.

SUPPORTING INFORMATION

APPENDIX A: Captive Management & Husbandry of Texas Horned Lizards, Oklahoma City Zoo

BASIC INDOOR HOUSING: Each setup consists of a 20 gallon-long glass aquarium with approximately 1.5–3 inches of locally sourced and sifted clay substrate (see later section on substrate sifting). Each aquarium is furnished with one rock structure under basking bulb to retain heat after lights cut off, one large artificial plant upright for shade, small aquarium plants sideways for ground cover, one piece of cholla wood for refuge, and a water bowl under each misting nozzle primarily to prevent dripping and substrate clumping (See images 1 and 2). **Upright cage furniture should be at least 3” from the top of the aquarium to ensure lizards and food items are not able to climb out.** Heating and light sources are provided by 100-watt basking bulbs (8:16 hr on/off cycles; 9:00–17:00) over each tank and T5 HO Reptisun 10.0 UVB lights (11:13 hr on/off cycles; 8:00–19:00) spanning three tanks each. All lighting is automated via timers. Basking temperatures under heat lamps should reach ~95–100℉ at surface level with ambient cool ends ~80℉. Ambient temperature in the Lizard Lab should be set to 78℉ year-round to achieve these temperatures. THL are within “ferguson zone 4”, meaning they need UVI within the 2.6 to 3.5 range, with a maximum of 4.5 to 9.5 in the basking zone. A RODI filtered water setup with a MistKing misting system is programmed to provide drinking water twice daily, 10:30 for 45 seconds and 14:00 for 35 seconds. Misting nozzles should remain aimed at tank sidewalls as water droplets seem preferred by lizards for drinking.

Supply List:

- 20 gallon-long glass or acrylic aquariums
- 100-watt basking bulb (Flukers generally preferred for price, any 100w basking bulb should work. Other wattages can be used by adjusting heights of heat lamps above tanks but 100w seems most consistent)
- 10.0 UVB T5 T0 UV bulbs (Zoomed Reptisun 46" 10.0 T5HO UVB Bulbs)
- Fine sifted local substrate from elephant pile
- Artificial plants (upright and aquarium style)
- Cholla wood
- Basking rocks (locally collected)
- Shallow water dish
- ROBuddy RODI system
- MistKing Advanced Misting system

Image 1 and 2: Tank Habitat Layout

SOCIAL GROUPINGS: Lizards at the OKC Zoo have been housed both individually and communally as hatchlings. From anecdotal experience, neither method leads to substantial differences in lizard sizes through the first year of life. Additional data may refute these generalized observations though. In our first attempt at housing hatchlings communally (four lizards/20-gallon tank), we split these juveniles into individual enclosures at ~9 months old to emulate when lizards would be emerging from brumation after their first winter and leaving natal sites. **Extra attention should be paid when feeding animals housed in groups to ensure all of them are able to eat as much food as they need. If one individual appears to be outcompeted, or is not gaining weight like the others, it should be separated from the group and/or group sizes reduced and the enclosure modified (adding basking rocks etc.) until all animals within an enclosure are gaining weight**

FEEDING AND DIET: THL diet in the Lizard Lab consists of commercially available Fruit flies (*Drosophila hydei* primarily, some *D. melanogaster*) as well as appropriately sized banded crickets (*Gryllodes sigillatus*) should make up the bulk of their diet. Hatchlings can be started on fruit flies and pinhead crickets. As they grow, the lizards should shift more towards larger crickets, bean beetles (*Callosobruchus maculatus*) and mealworms (*Tenebrio molitor*), with fruit flies becoming more of an enrichment item. The size of the cricket or other food item depends on the size of the lizard. **Generally, the cricket should be smaller than the lizard's head. If you are unsure of what size cricket to feed an animal, consult the Herpetology department liaison or the Curator/Assistant Curator of Herpetology.** All food is dusted with a Repashy Calcium Plus supplement and/or Repashy Formic-Cal Plus Ant Eater supplement prior to feeding. Feeding is done every other day at random times to simulate natural feeding tendencies. The number of prey items varies depending on prey species. In general, feed sufficient prey numbers that can be consumed by a lizard in ~15 minutes. **If unsure about quantity, consult the Herpetology department liaison or Curator/Assistant Curator of Herpetology. When lizards are first being started, they should be placed into a small bowl or dish with the food items to ensure easy capture. Most food items will immediately hide or burrow and therefore not be eaten. As lizards become hardier, they can be scatter-fed. Lizards should**

always be observed when eating to ensure they are eating well. If a lizard is not gaining weight as expected, or is losing weight, it should be fed inside a feeding bowl.
***Harvester ants can be fed on occasion but must be under the direction of the Herpetology department liaison or Curator/Assistant Curator of Herpetology. This is to ensure proper capture and feeding of the ants, as well as making sure the proper species is being fed.**

Supply List:
Fruit flies
Banded Crickets
Bean Beetles
Mealworms
Repashy Calcium Plus
Repashy Formic-Cal

SOAKING: Hatchling and juvenile THLs are soaked bi-weekly for 30–60 minutes to ensure proper hydration. Lizards are soaked individually or as a group (depending on current housing situations) in 6 qt tubs placed directly inside each home enclosure (See images 3 and 4). Each THL is caught and placed in one of the tubs and the plants/drinking dishes are removed from the tanks. The 6 qt tub is then placed inside of the aquarium but away from the heat lamp. Lukewarm water (~85℉) is then added to each tub with just enough to cover the bottom but not so much that the lizard is submerged. This species uses capillary action to drink and therefore does not need very deep water. Water levels should NEVER be above THL heads.

Watch for gulping to ensure the lizards are drinking and pay careful attention to their heads remaining above water so they do not drown. Once soaking is completed, each THL needs to be removed from the water and returned to the enclosure. Shed skin may be removed carefully with tweezers to prevent loss of circulation in the tail and digits. These opportunities can also be used to reapply identifying marks (i.e., nail polish) as needed. Take careful consideration to avoid inverting lizards for long periods, causing stress and labored breathing. **Discard water after soaking, sanitize with VEDCO D-256 and dry tubs for the next use.**

Supply List:
Clean 6 qt tub
Lukewarm water (~85℉)
Paper towels
Tweezers
VEDCO D-256

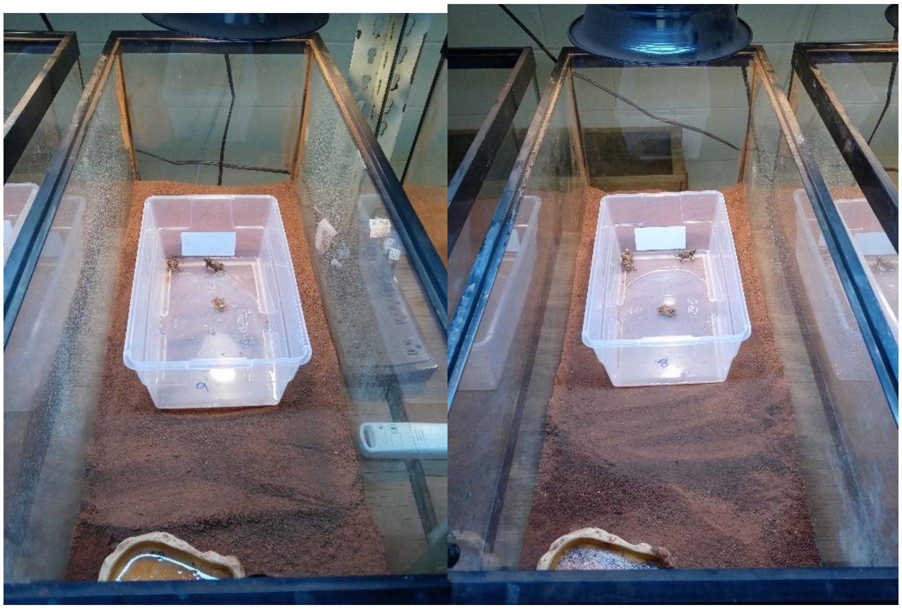

Image 3 and 4: THL soaking under heat lamp

WEIGHING: Weighing is done bi-weekly and usually in conjunction with soaking and drying each THL so as to avoid unnecessary additional interactions with lizards. Weight is recorded in grams and logged into the records kept in Dropbox. Putting the scale inside a 6 qt tub to weigh THLs helps ensure they do not escape.

Supply List:
Digital 0.01-gram Palmscale
6 qt tub

FECAL SAMPLING: Fecal samples are collected weekly during the active season (rarely if ever during the inactive winter season) in a sterile 6 qt tub. Tubs are sterilized by spraying a 10% bleach solution in each after its usage and allowing it to dry. Each juvenile is put into a tub weekly and offered food in the tub for approximately 45 minutes. Any fecal deposits are collected with sterilized tweezers and stored immediately in DNA/RNA Shield vials. All collected samples should be transported monthly to the Sam Noble Museum for curation and storage.

Supply List:
Sterile 6 qt tub
Sterile vials 1.5 mL cryovials
Zymo DNA/RNA Shield
Sterile tweezers
Bleach and hand mister to sterilize stubs and tools

BRUMATION: In their natural environment, THLs are usually dormant from October to April. Brumation is not stimulated in the Lizard Lab currently though THLs do decrease their activity significantly during the winter. **This is despite consistent light cycles and stable temperatures year-round. If wishing to brumate the lizards over winter consult with the Herpetology department liaison or the Curator/Assistant Curator of herpetology beforehand.**

IDENTIFICATION & SEXING: Individual THLs can be identified by differences in the ventral patterns (spot patterns) though we use water-based nail polish and dorsal spots for each lizard housed communally. Patterns on each animal are described in the lizard weight database. Individuals can be sexed at roughly one year old via femoral pores and hemipenile bulges. Males have a distinct broadening at the base of their tail with enlarged femoral pores, and females have tails that rapidly narrow in a more pronounced "V" shape with less defined femoral pores. See images 4 and 5.

Image 5: Female *Phrynosoma cornutum*

Image 6: Male *Phrynosoma cornutum*

SPOT CLEANING TANKS: Spot cleaning of tanks should occur weekly to ensure healthy living environments for animals. To do so, remove everything from the tank except the THLs and place tank items into a separate tank or storage bin in case there is a stowaway THL in tank accessories. Take a small plastic cup and tweezers, remove all visible fecal matter, urates, shed skin and other debris from the tank and discard. No fecal matter should be visible on the top of the soil when done. When done, rearrange the tank as was setup previously. Also take this opportunity to do daily headcounts and complete visual inspections of THLs.

FULL TANK CLEANING: Full tank cleanings are generally done monthly. For these more intensive efforts, remove all decorations from the tank and place them in a separate container. Remove all lizards from the tank as well and place them in their own container. All substrate in the tank should then be resifted through the mesh strainers in the Lizard Lab "substrate tools" box. This sifting will remove any remaining feces, urates, and miscellaneous debris in the tanks. Once all substrate has been resifted, tanks can be redecorated, and lizards replaced in their tanks. Substrate should be fully replaced with fresh substrate at least every 6 months.

SIFTING SAND FOR TANKS: Clay dirt is generally placed on the hill adjacent to the Lizard Lab once every summer for sifting and substrate replacement at the request of the Lizard Lab technician. Contact Dr. Brad Lock to organize dirt acquisition. In general, once clay substrate is collected it will need to be sorted twice. Once with the course black colander in a first pass to remove large rocks, leaf litter, etc. Once all dirt has been sifted once coarsely, a second pass is needed with fine mesh screens to produce the desired sand particle size. Each 20-gallon aquarium should be filled with 1.5–3 inches of this double-sifted substrate as previously described.

DAILY HUSBANDRY DUTIES:

- Head count (visual inspection of all THLs)
- UVB light check (make sure they are on and functioning)
- Heat lamp check (make sure they are on and functioning)
- Lizard Lab temperature checks (ambient and basking temps)
- Check and top off water bucket for misting system if needed (water level should stay around lettering on bucket, **will add mark to bucket for clarification**) ‘
- Feeding every other day according to the schedule in the Lizard Lab
- Check prey item cultures to ensure sufficient food is available for coming week(s)

WEEKLY HUSBANDRY DUITES:

- Soaking every other week
- Weighing every other week
- Spot clean tanks weekly according to the schedule

MONTHLY HUSBANDRY DUTIES:

- Full tank cleaning
- Sweep/wipe down Lizard Lab
- Inventory supply closet
- Cloacal swaps
- Fecal samples

Reference Figures of Lizard Lab Setup: A) Water filtration system setup; B) Water filtration system close-up; C) Left counter “feeding station”; D) MistKing timer; E) General rack/tank layout; F) Supply closet (right); G) Supply closet (left); H) Heat lamp timer; and I) UV lights timer.

RELEVANT LITERATURE ON TEXAS HORNED LIZARDS:

Can Specialists Generalize? Diets of Texas Horned Lizards (*Phrynosoma cornutum*) in small Texas Towns. https://repository.tcu.edu/bitstream/handle/116099117/21824/Alenius_tcu_0229M_10900.pdf?sequence=1&isAllowed=y

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Colorado Parks & Wildlife. (2025). Texas horned lizard. Available at: https://cpw.state.co.us/species/texas-horned-lizard (Accessed January 11, 2025).

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Rhoads D. D., Williams D. A. (2023). Background color matching in Texas horned lizards (*Phrynosoma cornutum*). J. Herpetol. 57, 27–35. doi: 10.1670/22-008.

Vesy, M. N., J. L. Watters, R. W. Moody, E. M. Schauber, J. M. Mook, and C. D. Siler. 2021. Survivorship and Spatial Patterns of an Urban Population of Texas Horned Lizards. The Journal of Wildlife Management 85:1267–1279. https://wildlife.onlinelibrary.wiley.com/doi/full/10.1002/jwmg.22064

CLOSET INVENTORY:	**LAB IN-USE SUPPLIES:**
(~30) red sandstone (~10) concrete hides (~12) 36” wooden stakes (1) Bag of hatching medium (1) Small tub of wet silica (1) Cup of dry silica (1) Tub of hatching materials	(36) 20-gallon long aquarium tanks (29) Heat lamps (12) UV light fixtures with bulbs (36) Mist heads and corresponding misting tubes (4) Timers (2) 5-gallon buckets (One in use for misting and other is not in use)

(~35) Cholla wood
(1) Cardboard paper towel roll
(13) Medium shallow water dishes
(19) Small shallow water dishes
(~10) Egg crates
(~15) Hefty 33-gallon black trash bags
(1) Air duster with box and accessories
(~24) Artificial long stem aquatic plants
(1) 8oz Goo Gone
(4) Small white plastic soaking dishes
(~20) Rectangle plastic tubs
(3) Small white plastic soaking tubs
(1) Super Stater Kit for automatic feeder setup
(1) Razor blade
(~6) Misc. plastic water filter tools/parts
(2) Replacement cartridges for water filter (order DI cartridge)
(2) White Styrofoam incubators
(29) Small black hides
(37) Medium grey hides
(26) Medium brown tiles
(13) Small gray tiles
(~12) Black UV power cords
(~12) White LED power cords and associated parts
(3) Surge protectors
(12) Unboxed stock UV bulbs
(2) Unboxed 110W bulbs
(2) Unboxed Ceramic heat bulbs
(15) Basking Bulbs in the box (Fluker's)
(3) Brown paper towels
(27) Aquarium tank lids
(~13) T5 UV lights (Always order extra to make-up for broken ones)
(~30) Clear 6-quart tubs
(7) 8ft extension cords
(2) 30ft extension cords
(1) Surge protector
(1) White Duct tape
(1) Mist King box with extra mist tubing inside
(1) Box of nails
(1) Roll of metal siding
(1) Roll of garden liner
(1) 1 gallon pressure sprayer

(1) DI filter
(1) RO Buddy filtration system (Recommend just buying medium in the future)
(1) Sediment Cartridge
(1) Mist King system with timer
(4) Surge protectors (white for UV fixtures/ black for heat lamps)
(1) Small step ladder
(2) Racks (3 shelves each)
(2) 72-quart tubs
(1) Large white incubator
(1) Powdered calcium supplement

DRAWER CONTENTS:
(1) Box of blue gloves
(1) Cup of egg shells
(~9) Tinker AFB trackers
(1) Bundles of twist ties
(1) Lighter
(1) Small box of q-tips
(1) Bag of zip ties
(1) Roll of black electrical tape
(1) Dry erase marker
(1) Hole puncher
(2) Box of Command Stripes
(1) Box of assorted nail polishes
(1) Marker
(2) Batteries (AAA and AA)
(1) Box of pens
(1) Box of hooks
Various instruction manuals
Temperature gun
UV sensor
Note pad
Computer Monitor
Mac Air Laptop

Supplementary Table S1: Detailed survival data over the study period (2023 – 2024) for 2 age classes of headstarted and resident Texas Horned Lizards on Tinker Air Force Base, OK, USA.

ADULTS

ID	Origin	Sex	Start Date	End Date	Days	Status	Note
1126	H	F	5/25/2023	6/12/2023	18	0	Dropped tag. Last: 6/11. Found: 6/13.
1127	H	F	5/25/2023	5/11/2024	352	0	Tag failure or left reserve. Last: 5/10.
1128	H	M	5/25/2023	8/16/2023	83	1	Dropped tag. Possible mammalian. Last: 8/15. Found:8/17
1129	H	F	5/25/2023	8/16/2023	83	1	Dropped tag. Possible mammalian. Last: 8/15. Found:8/17
1130	H	M	5/25/2023	6/3/2023	9	0	Dropped tag. Last: 6/2. Found: 6/7. Tag stop: 6/5.
1132	H	M	5/25/2023	8/12/2023	79	0	Dropped tag. Shed. Last: 8/11. Found 8/15.
1133	H	M	5/25/2023	8/2/2023	69	0	Dropped tag. Shed. Last: 8/1. Found: 8/3.
1134	H	F	5/25/2023	8/30/2023	97	1	Dropped tag: Mammalian. Last: 8/29. Found: 8/30.
1135	H	M	5/25/2023	8/16/2023	83	1	Dropped tag. Possible mammalian. Last: 8/15. Found:8/17
1136	H	M	5/25/2023	6/21/2023	27	1	Depredated: Snake. Last: 6/20. Found: 6/22.
1137	H	F	5/25/2023	6/7/2023	13	1	Dropped tag. Possible avian. Last: 6/6. Found: 6/7.
1083	H	M	5/25/2023	7/31/2024	433	1	Found partially decapitated/ face removed. Euthanized same day. Found: 7/31
1087	H	M	5/25/2023	8/16/2023	83	1	Dropped tag. Possible mammalian. Last: 8/15. Found:8/17
1088	H	F	5/25/2023	8/16/2023	83	1	Dropped tag. Possible mammalian. Last: 8/15. Found:8/17
1089	H	M	5/25/2023	4/3/2024	303	1	Infection of cloaca. Euthanized.
1091	H	F	5/25/2023	6/18/2024	390	1	Predated, decapitated with arm off. Marks on transmitter. Last: 6/18. Found: 6/19.
1051	R	M	4/12/2023	5/1/2023	19	1	Depredated: Snake. Last: 4/28. Found: 5/3.
1076	R	F	4/3/2023	5/7/2023	34	1	Natural death: Dried carcass. Last: 4/28. Found 5/16.
1112	R	M	5/1/2023	4/23/2024	358	0	VHF signal loss.

1121	R	F	4/12/2023	5/26/2024	410	1	Found decapitated. Last: 5/24. Found: 5/27.
1122	R	F	4/14/2023	8/8/2023	116	1	Dropped tag. Possible mammalian. Last: 8/7. Found: 8/23.
1140	R	F	4/20/2023	7/2/2024	439	1	Found decapitated. Last: 7/1. Found: 7/2/2024.
1141	R	F	4/19/2023	5/15/2024	392	0	VHF signal loss.
1142	R	M	4/3/2023	10/1/2024	547	0	*End of study.*
1143	R	M	4/5/2023	6/27/2024	449	0	VHF signal loss.
1144	R	F	4/10/2023	7/1/2023	82	1	Dropped CTT tag. Last: 6/30. Found: 7/3.
1145	R	M	5/2/2023	6/7/2024	402	1	Found decapitated. Last: 6/4. Found: 6/10.
1146	R	M	5/18/2023	5/11/2024	359	0	VHF signal loss.
1147	R	M	5/25/2023	9/22/2023	120	1	Dropped CTT tag. Last: 9/21. Found: 9/27. No silicon on tag,
1148	R	F	5/30/2023	10/1/2024	490	0	*End of study.*
1149	R	M	5/30/2023	8/18/2023	80	0	CTT signal loss. Last: 8/17.
1150	R	F	5/31/2023	4/26/2024	331	1	Unknown cause.
1151	R	F	5/31/2023	10/1/2024	489	0	*End of study.*
1152	R	M	6/7/2023	7/27/2024	416	0	Shed.
1154	R	M	7/26/2023	6/15/2024	325	0	CTT signal loss.
JUVENILES							
1164	R	F	5/20/2024	9/14/2024	117	1	Found decapitated. Last: 9/12. Found: 9/16.
1165	R	F	5/20/2024	10/1/2024	134	0	*End of Study*.
1166	R	F	5/28/2024	6/26/2024	29	1	Transmitter attached to bone. Completely consumed. Last: 6/25. Found: 6/28
1167	R	F	6/4/2024	9/22/2024	110	1	Chewed transmitter. Possible Coyote. Last: 9/19. Found: 9/24.
1168	R	F	6/6/2024	10/1/2024	117	0	*End of Study.*
1169	R	F	6/7/2024	6/25/2024	18	1	VHF Found: 6/25, Found decapitated. Last: 6/24. Found: 6/26.
1170	R	F	6/7/2024	10/1/2024	116	0	*End of Study.*
1171	R	M	6/7/2024	7/24/2024	47	0	Tag failure. Last: 7/24. Left study site.
1172	R	M	6/14/2024	10/1/2024	109	0	*End of study.*
1155	H	F	5/20/2024	7/31/2024	72	1	Found decapitated. Last: 7/30. Found: 7/31.

1156	H	M	5/20/2024	9/5/2024	108	1	Found w/ broken jaw. Maybe from zoo volunteers. Euthanized. Last: 9/3. Found: 9/5.
1157	H	F	5/20/2024	6/9/2024	20	1	Found decapitated. Last: 6/8. Found: 6/10.
1158	H	F	5/20/2024	10/1/2024	134	0	*End of study.*
1159	H	F	5/20/2024	6/12/2024	23	1	Predation, transmitter only. Last: 6/11. Found: 6/13.
1160	H	F	5/20/2024	6/25/2024	36	1	Found decapitated. Last: 6/24. Found: 6/25.
1161	H	M	5/20/2024	6/12/2024	23	1	Predation, transmitter only. Last: 6/11. Found: 6/13.
1162	H	M	5/20/2024	8/4/2024	76	0	Left study site. Last: 8/3.

Supplementary Table S2. Sample size by sample type and month before and after rarefaction and filtering processes.

Sample Type	**Month**	**Sample no. (pre-rarefaction and filtering)**	**Sample no. (post-rarefaction and filtering)**
Headstart Gut Microbiome	January	22	14
	February	29	15
	March	30	16
	April	26	12
	May	13	13
	June	13	12
	July	11	9
	Total	**144**	**91**
Resident Gut Microbiome	May	11	11
	June	3	2
	July	12	7
	Total	**26**	**20**
Diet Microbiome	January	9	9
	February	7	7
	March	6	6
	April	6	6
	May	7	6
	June	6	6
	July	8	8
	Total	**49**	**48**
Environment Microbiome	January	8	8
	February	8	8
	March	8	8
	April	9	8
	May	8	8
	June	8	8
	July	7	7
	Total	**56**	**55**

Supplementary Table S3. Mean Phyla relative abundance by sample type. The category "other" represents the represents the summed relative abundance of phyla not present at a relative abundance of at least 0.05 in one sample.

Phylum	Captive Headstart	Post-Release Headstart	Resident	Captive Diet	Wild Diet	Captive Environment	Wild Environment
Acidobacteriota	0.009307887	0.065235746	0.047241364	0.000134159	0.010215491	0.060302501	0.140081943
Actinobacteriota	0.150577633	0.326291832	0.321417895	0.024835478	0.038980379	0.131038171	0.167921428
Bacteroidota	0.044223606	0.058247712	0.035096768	0.065155129	0.063874177	0.073582638	0.07711887
Bdellovibrionota	0.00031226	0.002264967	0.001647239	1.20E-06	0.000252593	0.003114148	0.004630875
Chloroflexi	0.027995464	0.021806338	0.016229355	0.00023606	0.003877677	0.099629084	0.039463099
Cyanobacteria	0.004526031	0.011934748	0.014457535	0.124762368	0.116984292	0.003558479	0.062348603
Deinococcota	0.004476977	0.068058158	0.115838396	5.81E-05	0.000167133	0.005860382	0.008069595
Desulfobacterota	0.001812348	0.000514161	0.000212598	0.001382228	0.000907005	0.001364842	0.000616813
Firmicutes	0.188872829	0.084238952	0.092666112	0.411279811	0.04926389	0.186669886	0.021762046
Fusobacteriota	0.001066112	0.000102798	0	0.003232256	0	5.55E-06	2.56E-06
Gemmatimonadota	0.003187651	0.004566279	0.003549738	8.65E-06	0.00054786	0.016807385	0.011208574
Methylomirabilota	0.000200595	0.002635427	0.001025459	0	7.87E-05	0.000327515	0.003227059
Myxococcota	0.003903962	0.017210831	0.010095297	3.50E-05	0.021581387	0.016316029	0.040417353
NB1.j	0.000874601	8.50E-05	4.14E-06	0	7.85E-06	0.002899544	0.000275756
Other	0.002591005	0.00646318	0.00480103	0.000122555	0.000936362	0.012212731	0.013094631
Planctomycetota	0.009568878	0.019074743	0.013221091	0.000135879	0.00312913	0.062183004	0.053564428
Proteobacteria	0.542768842	0.298797414	0.314503177	0.35553109	0.684857973	0.306899142	0.322694882
Verrucomicrobiota	0.003733313	0.012471723	0.007992805	0.013089986	0.004338103	0.017228957	0.033501505

Supplementary Table S4. Multiple comparison post-hoc of the Linear Mixed Model testing the effect of month on headstart lizard gut microbiome Shannon Diversity.

Contrast	Estimate (s.e.)	df	T-ratio	p -value
January – February	0.3717 (0.530)	71.8	0.701	0.9921
January – March	0.0285 (0.521)	70.9	0.055	1
January – April	1.2354 (0.563)	74.6	2.195	0.3109
January – May	-2.7661 (0.550)	73.6	-5.026	**0.0001**
January – June	-1.7022 (0.562)	72.9	-3.031	0.0502
January – July	-0.6074 (0.613)	76	-0.991	0.9544
February – March	-0.3432 (0.512)	70.1	-0.671	0.9938
February – April	0.8636 (0.554)	73.6	1.56	0.7078
February – May	-3.1378 (0.541)	72.7	-5.801	**<.0001**
February – June	-2.0740 (0.553)	73.6	-3.748	**0.0062**
February – July	-0.9791 (0.605)	76.6	-1.618	0.6711
March – April	1.2068 (0.545)	72.7	2.214	0.3012
March – May	-2.7946 (0.532)	71.8	-5.249	**<.0001**
March – June	-1.7308 (0.545)	72.7	-3.176	**0.0341**
March – July	-0.6359 (0.597)	75.6	-1.065	0.9362
April – May	-4.0014 (0.574)	75.6	-6.977	**<.0001**
April – June	-2.9376 (0.584)	75	-5.028	**0.0001**
April – July	-1.8427 (0.634)	78.4	-2.906	0.0682
May – June	1.0638(0.572)	73.9	1.859	0.5134
May – July	2.1587 (0.621)	75.4	3.474	**0.0143**

Supplementary Table S5. Multiple comparison post-hoc of the Linear Mixed Model testing the effect of month on headstart lizard gut microbiome OTU richness.

Contrast	Estimate (s.e.)	df	T-ratio	p -value
January – February	203.8 (139)	71.5	1.462	0.7657
January – March	-89.1 (137)	70.8	-0.65	0.9948
January – April	353.4 (148)	74.2	2.385	0.2197
January – May	-1080.0 (145)	73.3	-7.457	**<.0001**
January – June	-465.2 (148)	72.6	-3.148	**0.0368**
January – July	17.4 (161)	75.6	0.108	1
February – March	-292.9 (134)	70	-2.178	0.3206
February – April	149.5 (146)	73.2	1.026	0.9462
February – May	-1283.8 (142)	72.4	-9.021	**<.0001**
February – June	-669.0 (146)	73.3	-4.594	**0.0003**
February – July	-186.4 (159)	76.1	-1.17	0.9032
March – April	442.4 (143)	72.4	3.085	**0.0436**
March – May	-990.9 (140)	71.6	-7.076	**<.0001**
March – June	-376.1 (143)	72.5	-2.623	0.1342
March – July	106.5 (157)	75.2	0.677	0.9935
April – May	-1433.4 (151)	75.2	-9.492	**<.0001**
April – June	-818.6 (154)	74.6	-5.322	**<.0001**
April – July	-335.9 (167)	77.9	-2.01	0.4168
May – June	614.8 (151)	73.6	4.082	**0.0021**
May – July	1097.4 (164)	75	6.709	**<.0001**

Supplementary Table S6. Pairwise Alpha Diversity Kruskal-Wallis test results. Significant values are bolded (p or q < 0.05). Q-value is adjusted p-value (Benjamini & Hochberg correction).

Metric	**Groups**	**H**	**p-value**	**q-value**
Shannon Diversity	May Headstart v. May Resident	14.843916	**0.00012**	0.00012
	July Headstart v. July Resident	2.0420168	0.15301	0.15301
	OKC Zoo Prey Items v. TAFB Prey Items	0.231341	0.63053	0.63053
	OKC Zoo Environment v. TAFB Environment	4.404794	**0.03584**	0.03584
OTU Richness	May Headstart v. May Resident	12.706714	**0.00036**	0.00036
	July Headstart v. July Resident	0.0028011	0.95779	0.95779
	OKC Zoo Prey Items v. TAFB Prey Items	1.0716542	0.30057	0.30057
	OKC Zoo Environment v. TAFB Environment	0.094332	0.75874	0.75874

Supplementary Table S7. Texas Horned Lizard Beta Diversity PERMANOVAs results. Significant results are bolded (P < 0.05).

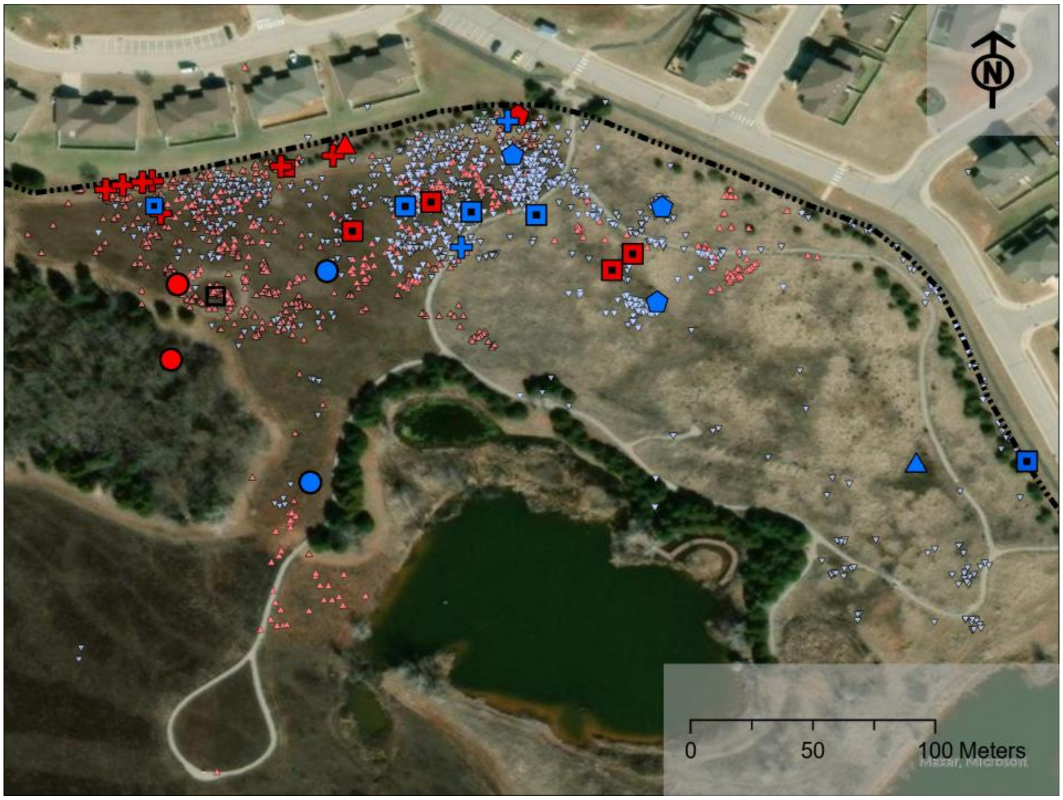
Metric	**Groups**	**pseudo-F**	**p-value**
Unweighted UniFrac Distance	May Headstart v. May Resident	1.807961	**0.006**
	July Headstart v. July Resident	1.13137	0.176
Weighted UniFrac Diversity	May Headstart v. May Resident	1.998213	**0.025**
	July Headstart v. July Resident	1.527538	0.107

Supplementary Table S8. All Sample Pairwise Unweighted UniFrac Distance PERMANOVAs results. Significant values are bolded (P < 0.05). Q-value is adjusted p-value (Benjamini & Hochberg correction).

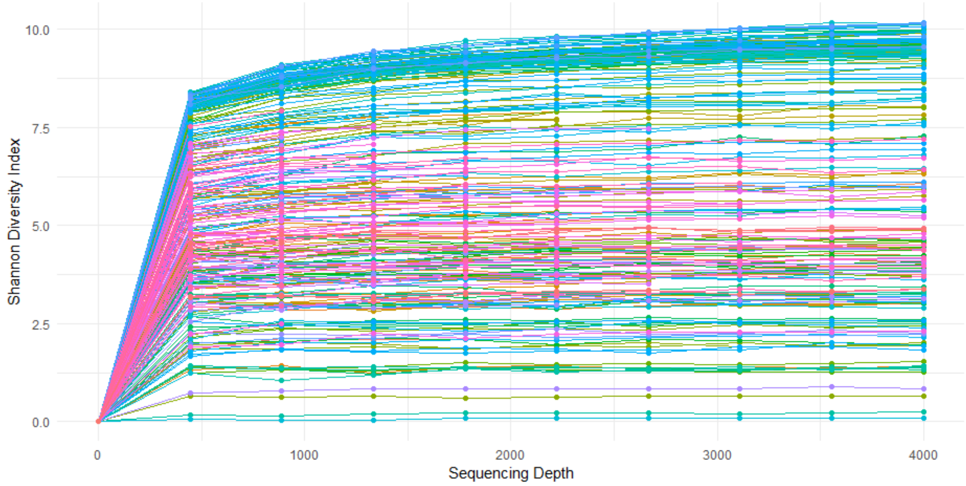
Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**q-value**
Diet Post Release	Diet Pre Release	48	999	3.968164519	**0.001**	0.00105
Diet Post Release	Environment Post Release	43	999	17.60085786	**0.001**	0.00105
Diet Post Release	Environment Pre Release	52	999	22.77801053	**0.001**	0.00105
Diet Post Release	Headstart Post Release	54	999	13.72358891	**0.001**	0.00105
Diet Post Release	Headstart Pre Release	77	999	9.594685625	**0.001**	0.00105
Diet Post Release	Resident	40	999	9.259111059	**0.001**	0.00105
Diet Pre Release	Environment Post Release	51	999	32.66090367	**0.001**	0.00105
Diet Pre Release	Environment Pre Release	60	999	39.446486	**0.001**	0.00105
Diet Pre Release	Headstart Post Release	62	999	28.32451969	**0.001**	0.00105
Diet Pre Release	Headstart Pre Release	85	999	18.65354071	**0.001**	0.00105
Diet Pre Release	Resident	48	999	19.37699697	**0.001**	0.00105
Environment Post Release	Environment Pre Release	55	999	17.42146193	**0.001**	0.00105
Environment Post Release	Headstart Post Release	57	999	6.200903102	**0.001**	0.00105
Environment Post Release	Headstart Pre Release	80	999	20.4648589	**0.001**	0.00105
Environment Post Release	Resident	43	999	6.610180916	**0.001**	0.00105
Environment Pre Release	Headstart Post Release	66	999	20.19354195	**0.001**	0.00105
Environment Pre Release	Headstart Pre Release	89	999	17.91750666	**0.001**	0.00105
Environment Pre Release	Resident	52	999	16.6570175	**0.001**	0.00105
Headstart Post Release	Headstart Pre Release	91	999	14.33458922	**0.001**	0.00105
Headstart Post Release	Resident	54	999	1.426025275	**0.042**	0.042
Headstart Pre Release	Resident	77	999	8.911188582	**0.001**	0.00105

Supplementary Table S9. All Sample Pairwise Weighted UniFrac Distance PERMANOVAs results. Significant values are bolded (P < 0.05). Q-value is adjusted p-value (Benjamini & Hochberg correction).

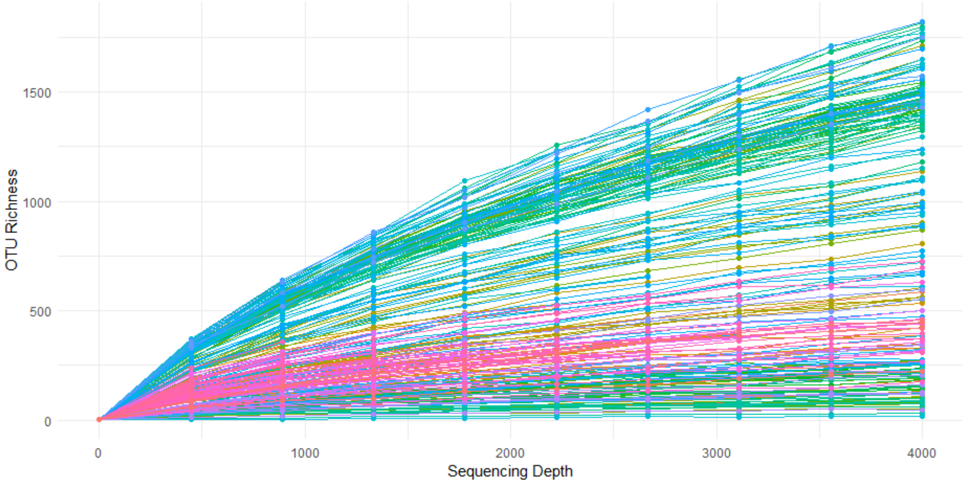
Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	**p-value**	**q-value**
Diet Post Release	Diet Pre Release	48	999	4.269696283	**0.001**	0.00105
Diet Post Release	Environment Post Release	43	999	22.96028984	**0.001**	0.00105
Diet Post Release	Environment Pre Release	52	999	32.34637363	**0.001**	0.00105
Diet Post Release	Headstart Post Release	54	999	17.98077469	**0.001**	0.00105
Diet Post Release	Headstart Pre Release	77	999	7.734463099	**0.001**	0.00105
Diet Post Release	Resident	40	999	9.657857005	**0.001**	0.00105
Diet Pre Release	Environment Post Release	51	999	30.31867669	**0.001**	0.00105
Diet Pre Release	Environment Pre Release	60	999	33.28202119	**0.001**	0.00105
Diet Pre Release	Headstart Post Release	62	999	22.68731197	**0.001**	0.00105
Diet Pre Release	Headstart Pre Release	85	999	10.75025655	**0.001**	0.00105
Diet Pre Release	Resident	48	999	12.73744905	**0.001**	0.00105
Environment Post Release	Environment Pre Release	55	999	45.14590038	**0.001**	0.00105
Environment Post Release	Headstart Post Release	57	999	18.66201094	**0.001**	0.00105
Environment Post Release	Headstart Pre Release	80	999	47.86294347	**0.001**	0.00105
Environment Post Release	Resident	43	999	18.9546018	**0.001**	0.00105
Environment Pre Release	Headstart Post Release	66	999	31.53634067	**0.001**	0.00105
Environment Pre Release	Headstart Pre Release	89	999	41.06164405	**0.001**	0.00105
Environment Pre Release	Resident	52	999	29.51609231	**0.001**	0.00105
Headstart Post Release	Headstart Pre Release	91	999	25.32474011	**0.001**	0.00105
Headstart Post Release	Resident	54	999	2.179218956	**0.042**	0.042
Headstart Pre Release	Resident	77	999	13.77383272	**0.001**	0.00105



Supplementary Figure S1: Map showing the locations of known mortalities over the entire study period (2023 – 2024) for 2 age classes of headstarted and resident Texas Horned Lizards on Tinker Air Force Base, OK, USA. Mortalities are overlaid over small triangles representing pooled relocations. Red shapes indicate headstarted lizards, while blue shapes indicate residents. Large triangles signify snake predations, crosses are presumed mammalian predations, circles are predations from unknown origin, and pentagons correspond to either natural or unknown causes of death. The black square represents the soft release pen, and the black dotted line corresponds to the wooden fence that runs along the northern boundary of the reserve.



Supplementary Figure S2. Alpha rarefaction curves based on Shannon Diversity Index. Each curve represents a unique sample. The dataset was rarified to a sequence count of 500 for downstream analysis.



Supplementary Figure S3. Alpha rarefaction curve based on OTU Richness. Each curve represents a unique sample. The dataset was rarified to a sequence count of 500 for downstream analysis.